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# The Interaction between NS1B protein of Influenza B virus and the Ubiquitin-like modifier ISG15: Insights into a Unique Species Specific Property of the Virus

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**by**

**Haripriya Sridharan, M.Sc, B.Sc**

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# Dedication

To:

My parents, Usha and K.R. Sridharan

and my husband, K.J. Balakrishnan

for their unwavering love and support.

## Acknowledgements

I would like to thank my supervisor, Dr. Robert Krug, for his guidance throughout my graduate work and teaching me how to do good science. I would also like to thank all my dissertation committee members, Drs. Jon Huibregtse, Henry Bose, Arlen Johnson and Maria Croyle for their interest in my research and valuable input. To all past and present Krug lab members, especially, Drs. Anita Latham, Chen Zhao, Tina Hsiang, Rei-lin Kuo and graduate students, Meghana Malur, Jesper Marklund and J.W. Park, I would like to express thanks for making my lab experience an enjoyable one and sharing ideas and reagents with me. Time spent with friends, Devashree, Anitha, Sandhya, Gauri, Srikanth, Anand, Gayathri, Kavita, Harish, Pali, Swati, Sastry, Nandu, Madhavi, Neely, Shilpa, Rajani, Aruna, Vishy, Shrawan, Bindu, Raghav, Anahita, and Fanglei will always be remembered with fondness. My longtime friends since childhood, Sandhya, Sripriya, Ranjini, Lavanya and Usha have been wonderful support. I have been extremely fortunate to have a wonderful and loving family. My parents and brother have always encouraged me to pursue my dreams and I owe my accomplishments today to them. And to my husband, Bala, I cannot express enough thanks. His patience, love and faith in me were instrumental in my finishing this study.

# The Interaction between NS1B protein of Influenza B virus and the Ubiquitin-like modifier ISG15: Insights into a Unique Species Specific Property of the Virus

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Influenza B virus causes a respiratory disease in people with a compromised immune system. The NS1B protein of influenza B virus is essential for virus growth and plays a crucial role in inhibiting the anti-viral responses mounted by the infected host cell. The N terminal 104 amino acids of NS1B bind a cellular protein called ISG15. ISG15 is an interferon induced 'ubiquitin-like' protein, and upon interferon induction, is conjugated to hundreds of targets. It has been found that both ISG15 and its conjugation inhibit many viruses. The focus of the current study was to characterize the interaction between NS1B and ISG15. Study of a recombinant influenza B virus which encoded a mutant NS1B protein that is unable to bind ISG15 revealed that ISG15 is mis-localized in cells infected with wild type but not the mutant influenza B virus. Further, such a mutant virus is attenuated in growth as compared to wild type virus in human cell lines but is not attenuated in canine cell lines. This result led to the discovery of the species specific

nature of the interaction between NS1B and ISG15. Specifically, NS1B was found to bind ISG15 homologs from human and old world monkeys like Rhesus macaques and African green monkeys but not those from mouse or canines. These findings were extended by identifying the hinge between the N and C terminal domains of ISG15 as one of the major determinants of species specificity. These results highlight the importance of using human or primate cell culture models to study the effect of ISG15 on influenza B virus, and raises new possibilities on differences in the function of the ISG15 system in different species.

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# CHAPTER 1: Literature Review

## 1.1 INFLUENZA VIRUSES

Influenza is a common respiratory illness, seen in 5-20% of the U.S population. The three types of influenza viruses are A, B and C. While influenza A virus causes infection in a wide variety of mammals and birds, influenza B and C are mostly human viruses. However, recently influenza B was discovered in seals (Osterhaus, Rimmelzwaan et al. 2000) and influenza C has also been isolated from swine in China (Yuanji and Desselberger 1984). In humans, the common symptoms of influenza infection are cold, chills, fever, muscle pain, sore throat, headache, coughing, and general discomfort (http://www.cdc.gov/flu/). Although influenza virus infection causes mild symptoms, influenza A viruses can cause a pandemic, resulting in widespread death, like the 1918 'Spanish flu', in which approximately 40 million people died worldwide. Influenza A viruses mutate at a high rate; therefore the strains vary every year making lasting immunity impossible. The limited host range of influenza B virus ensures that pandemics do not occur. However, influenza B causes a disease in people with a weakened immune system, like very young children or very old people (http://www.cdc.gov/flu/).

## 1.2 STRUCTURE AND PROTEINS OF INFLUENZA VIRUS:

Influenza viruses belong to the Orthomyxoviridae family of RNA viruses. In addition to influenza A, B and C viruses, this family also contains the genus *Thogotovirus* (sometimes referred to as influenza D). Influenza viruses are enveloped, negative sense, segmented RNA viruses, whose genome comprises 8 RNA segments for influenza A and B viruses and 7 segments for influenza C virus (Lamb and Krug 2001). Influenza A and B viruses are indistinguishable from each other morphologically; however influenza C virus can be distinguished because of the different arrangement of its glycoprotein spikes (summarized from (Lamb and Krug 2001)). Influenza viruses are pleiomorphic, usually 50-12nm in diameter. Viruses amplified in bird eggs or in tissue culture are more uniform and spherical; however those isolated from animals and humans show more variation in shape and filamentous forms might occur (ICTVdB, ver. 4). The viral genes that determine the morphology of the virus are not known.

The structure of influenza B virus is shown in figure 1.1 (Lamb and Krug 2001). The outermost layer of influenza virion is the viral envelope, which is derived from the host plasma membrane through budding. From the envelope of the virus, glycoproteins that form spikes radiate outwards. In influenza A and B viruses, these spikes are made up of two viral glycoproteins, the rod shaped haemagluttinin (HA) and mushroom shaped neuraminidase (NA), encoded by the viral RNA segments 4 and 6 respectively. HA is required for receptor recognition (reviewed in (Skehel and Wiley 2000), (Whittaker 2001)) and release of viral ribonucleoproteins (RNPs) from the viral envelope into the cytoplasm of the host cell (Whittaker 2001). NA is responsible for viral release from the cell, at the final stage of budding (Lamb and Krug 2001).

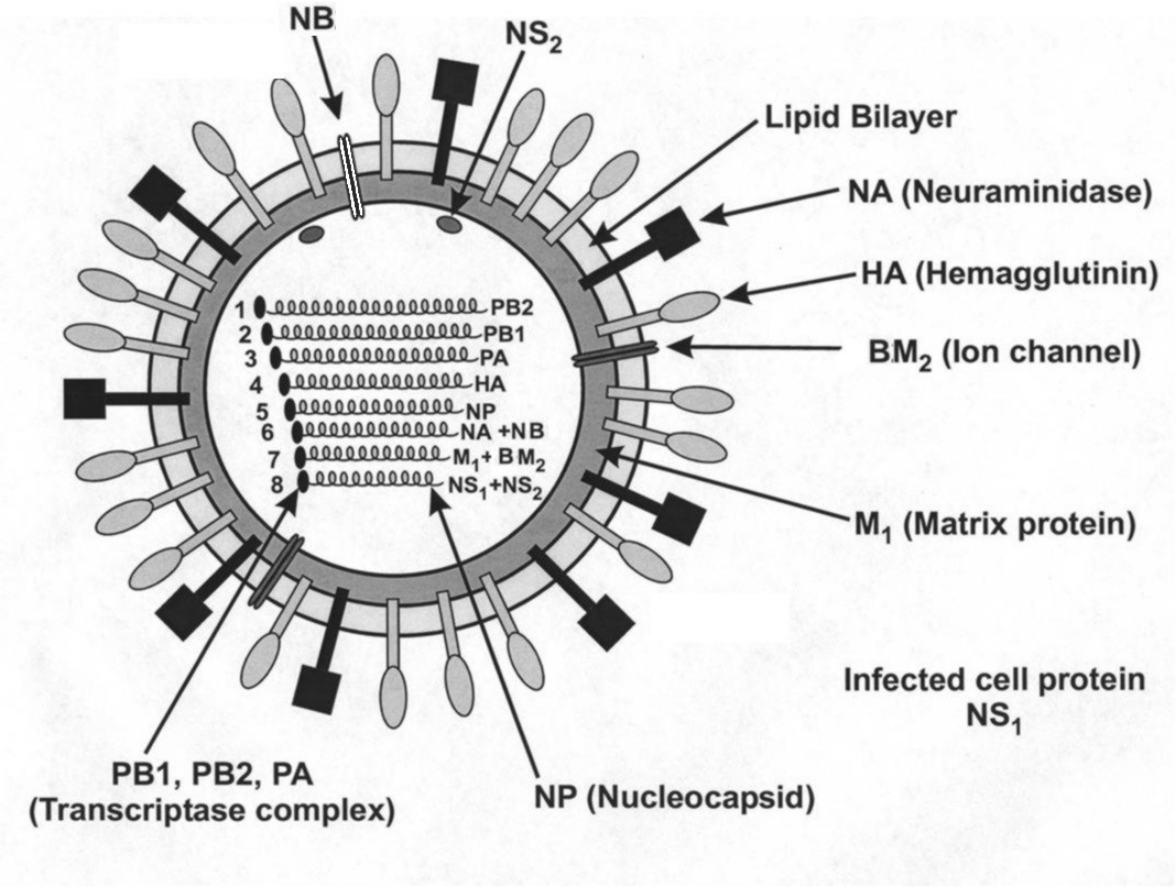



**Figure 1.1 Structure and proteins of influenza B virus.** The location of the structural proteins, viral envelope, matrix and eight genomic RNA segments are shown. The NS1B protein is present only in the infected cell. Adapted from Lamb and Krug 2001.

Besides playing important roles in the viral life cycle, HA and NA are also important antigenic determinants of the virus. Influenza C has a single glycoprotein, which combines all the above activities (Krug and Lamb 2001). This protein is called HEF, which stands for haemaglutinin, esterase and fusion. Another integral membrane protein present on the virion is the M2, BM2 or CM2 protein of influenza A, B and C viruses respectively. These proteins are encoded by the RNA segment 7 of influenza A and B viruses and segment 6 of influenza C virus. The M2 and BM2 proteins of influenza A and B viruses respectively are ion channel proteins which play important roles in the un-coating of the viral RNPs (Pinto and Lamb 2006). Influenza B virus contains another small integral membrane protein, called NB, which is encoded by the bicistronic segment 6, which also encodes NA. An equivalent of NB has not been discovered in influenza A virus, and its function has yet to be fully understood. Beneath the viral envelope lies the viral matrix protein (M1). The M1 protein is the most abundant protein present in the virion and forms connections with both the membrane bound HA and NA molecules as well as the core RNPs. Associated with the M1 protein is the nuclear export protein (NEP), also called the non-structural protein 2 (NS2), which is involved in the nuclear export of viral RNPs (O'Neill, Talon et al. 1998). The core of the virion is made up of viral RNP complexes, which contain the viral RNA segments, Nucleoprotein (NP), encoded by RNA segment 5 and the three subunits of the viral polymerase, PA, PB1 and PB2, encoded by the segments 3, 2 and 1 respectively. In the virion, each RNA segment is coated with the NP, and the ends are looped back and bound to the viral polymerase complex comprising of PB1, PB2 and PA proteins (figure 1.2).

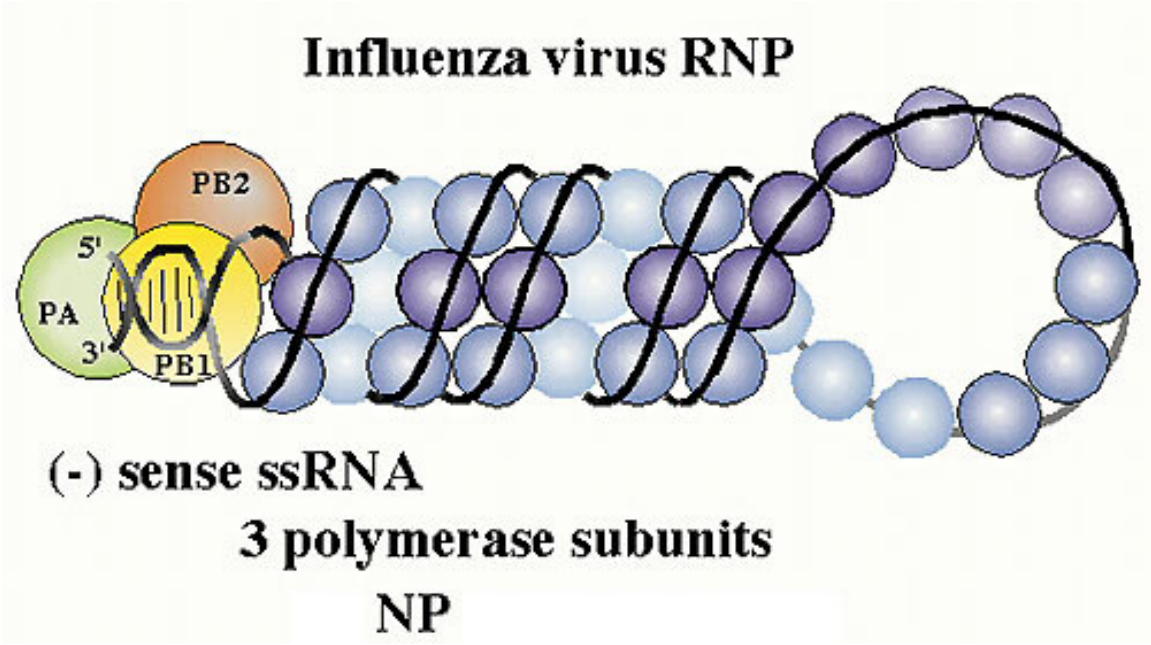



**Figure 1.2 Structure of influenza viral RNP in the virion.** Blue spheres represent NP monomers binding vRNA (black line). The vRNA is looped back and forms a hairpin structure with a duplex (of 5' and 3' ends of vRNA) which forms the binding site for the polymerase subunits. http://www.microbiologybytes.com/virology/Orthomyxoviruses.html

The RNA segments encode 12 and 11 proteins in influenza A and B viruses and 10 for influenza C. One viral protein, the non-structural protein 1 (NS1) is not present in the virion and is exclusively present in the infected cells of the host. NS1 is encoded by RNA segment 8 and is important to thwart the host's anti viral response. Another protein, PB2-F2, encoded by segment 1, has been found in cells infected with influenza A but not B virus. This protein is thought to be involved in apoptotic responses to infection (summarized from (Lamb and Krug 2001).

## 1.3 THE INFLUENZA VIRUS LIFE CYCLE

The influenza virus life cycle is depicted in figure 1.3 (Lamb and Krug 2001). Briefly, the virus attaches to receptors on the cell surface though it's HA, which results in endocytosis of the virus. Inside the endocytic vesicle, viral proteins HA and M2 mediate vesicle-viral membrane fusion, releasing the viral RNPs into the cytoplasm of the host cell, from where they are imported into the nucleus. The genome transcription and replication take place in the nucleus of the infected cell, involving the viral polymerase, NP and NS1 proteins. The newly assembled vRNPs are then exported from the nucleus by NEP and M1. Viral assembly is facilitated by the membrane proteins, HA, NA and M2 along with M1 and takes place in specialized regions of the plasma membrane called 'lipid rafts', which are concentrated in viral glycoproteins. Finally the virus is pinched off the plasma membrane of the infected cell by budding. Each of these steps in the viral life cycle is described in detail below.

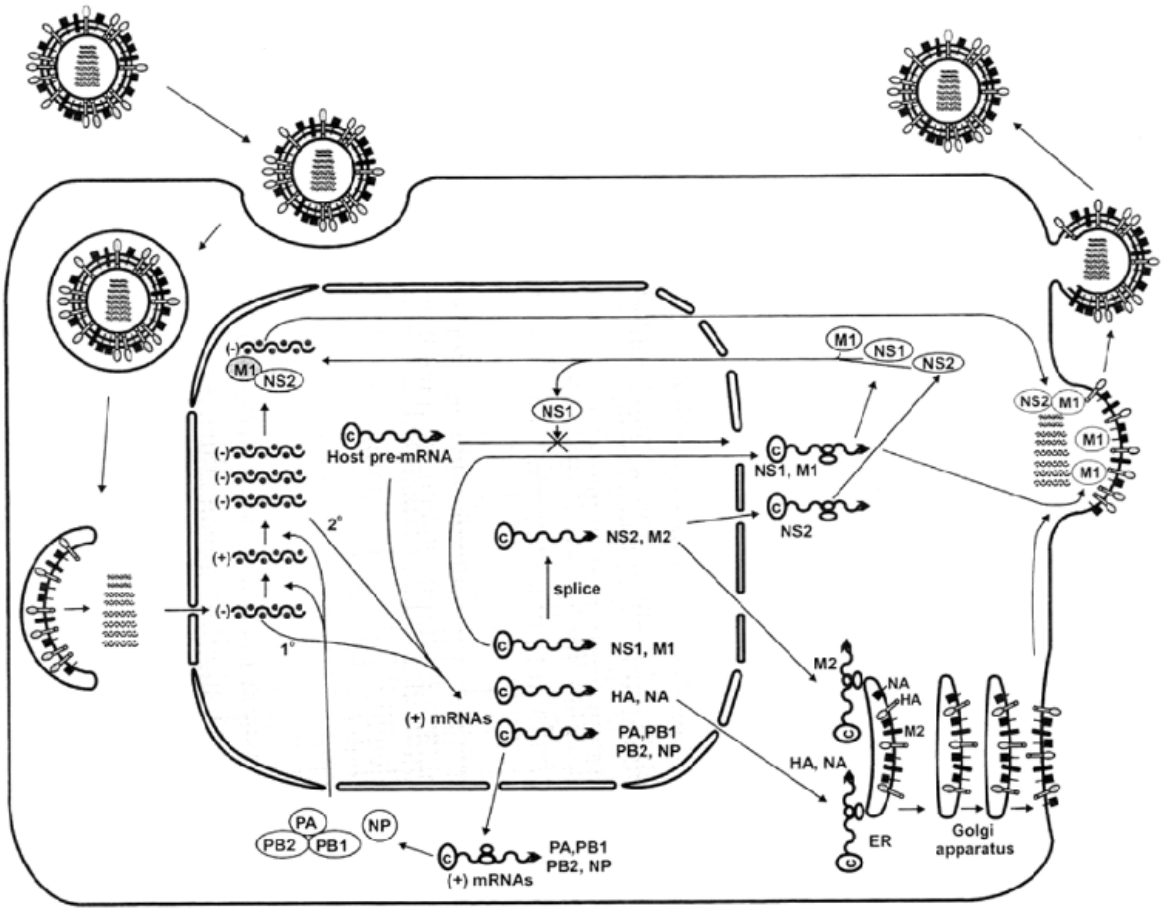



**Figure 1.3 Life cycle of influenza virus.** The virus is endocytosed into the cell, and in a pH dependent step, the vRNP is uncoated from the viral envelope. Replication takes place inside the nucleus. Virus assembly and budding take place in regions of the plasma membrane known as lipid rafts. See text for details. Adapted from Lamb and Krug 2001.

### *1.3.1 Virus binding to receptor*

The receptors for influenza virus is the terminal N acetyl neuraminic acid (sialic acid) present on glycolipids and glycoproteins. The viral protein responsible for recognizing and binding the receptors is the HA, which is present on the viral envelope. The HA molecule is synthesized as HA0 precursor peptide which is cleaved into HA1 and HA2 peptides. HA is a trimer, and can exist in three conformations: the un-cleaved HA0, the cleaved HA1 and HA2 at neutral pH and the pH induced altered conformation of HA1 and HA2 (summarized from (Lamb and Krug 2001)). The cleavage of HA is an essential step in the life cycle of the virus; in the absence of HA cleavage, a multiple cycle growth of influenza virus cannot be achieved (Lazarowitz and Choppin 1975), (Klenk, Rott et al. 1975). By recognizing the cellular receptors, HA also determines the host range of influenza viruses (reviewed in (Skehel and Wiley 2000), (Whittaker 2001)). The HAs isolated from different influenza A viruses differ in their ability to recognize different cellular receptors in which the sialic acid is either alpha (2,6) or alpha(2,3) linked. HA from influenza A viruses isolated from avian or equine sources recognize the alpha (2, 3) linked sialic acid chains, HA from human influenza A viruses recognize alpha (2, 6) linked chains and those isolated from swine recognize both (Ito 2000). HA from influenza B viruses (which predominantly infect only humans in nature) recognize alpha (2, 6) linked sialic acid. Glycans attached to aspargine 194 of these HAs prevent their binding to alpha (2,3) linked sialic acid chains. However when influenza B viruses are propagated in chicken eggs, the HAs lose the glycosylation sites at residues 194-196

leading to changes in receptor binding properties as well as antigenicity (Gambaryan, Robertson et al. 1999).

### *1.3.2 Virus un-coating and nuclear import*

After receptor attachment, the virus is endocytosed into the cells and is trafficked to an acidic endosomal vesicle. HA now has a second function, which is to bring about the fusion of the viral envelope with the endosomal envelope. This function is achieved by the conformational change of HA induced by the low pH. At this pH, the HA1 molecule remains mostly unaltered; however, the HA2 undergoes a folding event that exposes a previously buried 'fusion peptide' (Carr and Kim 1994), (Hernandez, Hoffman et al. 1996). The fusion peptide can intercalate into bilipid membranes, bringing about the fusion of the endosomal and viral envelopes, thus releasing the viral RNPs into the cytoplasm of the infected cell, from where they are imported into the nucleus (Whittaker 2001).

Another role of the low pH compartment is to facilitate the un-coating of the viral RNP complex from the encapsulating M1 protein. The M2 protein of influenza A virus is an ion channel protein (Pinto, Holsinger et al. 1992) that is highly specific for the conductance of protons (reviewed in (Pinto and Lamb 2006), (Lamb and Krug 2001), (Lamb Holsinger 1994), (Hay 1992) ). In the acidic endosomal vesicle, the passage of protons from outside to inside of the virion by M2 lowers the pH inside the virion, thereby disrupting the interaction between M1 and the underlying RNP complex (Lamb

Holsinger 1994), (Hay 1992). M2 is a type III integral membrane protein and in its native state, is a homo-tetramer (Sugrue and Hay 1991). The trans-membrane domains of the four monomers form the channel of the pore that conducts the protons. In the absence of un-coating of M1 mediated by M2, the RNP complex is too big to pass through the nuclear pore. The essential function of M2 is underscored by the fact that it is the target of an antiviral drug called amantadine. Influenza B virus also requires an essential low pH step in its life cycle and has an analogous ion channel protein called BM2 encoded by RNA segment 7. Like M2, BM2 is a type III, oligomeric (Balannik, Lamb et al. 2008), trans-membrane protein and has proton specific channel activity (Mould, Paterson et al. 2003). BM2 is also essential for proper replication of influenza B virus (Hatta, Goto et al. 2004). Another influenza B virus protein, the NB, was initially postulated to be analogous to influenza A virus M2. However, while NB is also incorporated into the viral envelope as a small integral membrane protein (Betakova, Nermut et al. 1996), and similar in size to M2, its ion channel activity has not been conclusively proven, neither is it essential for the virus replication (Hatta and Kawaoka 2003). The exact function of NB is still not clearly understood.

Once the RNP complex has been uncoated of M1, they are then imported into the nucleus through the nuclear pore complex. All the proteins in the RNP, namely, NP, PB1, PB2 and PA, contain nuclear localization signals (NLS) (O'Neill, Jaskunas et al. 1995), (Nieto, de la Luna et al. 1994), (Akkina, Chambers et al. 1987), (Jones, Reay et al. 1986), reviewed in (Whittaker and Helenius 1998).

### *1.3.3 Viral transcription and replication*

Influenza virus is unique among other negative sense segmented RNA viruses in that its replication takes place inside the nucleus rather than the cytoplasm. The viral RNA molecules are associated with the polymerase complex and the nucleocapsid protein (NP). Most of our understanding about the replication mechanisms of the polymerase have come from studies on influenza A virus polymerase.

The viral polymerase is an RNA dependent RNA polymerase and is a hetero-trimer of three subunits: PB1, PB2 and PA. PB1 is encoded by viral RNA segment 2, PB2 by RNA segment 1 and PA by RNA segment 3. The polymerase forms a complex in a linear fashion. PA binds the N terminus of PB1 through an extensive interaction of its C terminus and the C terminus of PB1 binds the N terminus of PB2 (Guu, Dong et al. 2008) (Gonzalez, Zurcher et al. 1996), (Zurcher, de la Luna et al. 1996), (Ohtsu, Honda et al. 2002), (Toyoda, Adyshev et al. 1996). Influenza polymerase is involved in two important reactions in the viral life cycle: transcription and replication.

***Transcription:***

Transcription involves the synthesis of *messenger* RNAs (mRNAs) from the negative sense *virion* RNA (vRNA) as a template. The viral mRNAs are similar in structure to the cellular mRNAs in that they are 5' capped and 3' poly-adenylated. The cap for the viral mRNA is obtained from the cellular mRNAs by a unique mechanism called 'cap snatching' (figure 1.4). In this reaction, the conserved 13 nucleotides at the 5' end of the vRNA binds to a site on the PB1 subunit, which activates the cap binding site on the PB2 and the endonuclease activity on PA subunits (Li, Rao et al. 2001), (Rao,

Yuan et al. 2003), (Yuan, Bartlam et al. 2009), (Dias, Bouvier et al. 2009). The endonuclease cleavage of the cellular mRNAs release capped structures that are 10-13 nucleotides in length which then serve as primers for mRNA synthesis. Although the 3P complex 'snatches' the cap from host mRNAs, the viral messages are saved from the same fate by the polymerase complex. By binding a conserved sequence in all viral mRNAs immediately downstream of the cap, a cap binding activity of the polymerase is activated whereby the caps are protected from cleavage (Shih and Krug 1996). The conserved 12 nucleotides at the 3' end of the vRNAs bind PB1 and serve as templates for mRNA synthesis. The viral mRNAs are elongated until the polymerase reaches a stretch of uridines, around 15 to 22 nucleotides from the 5' ends of the vRNA. At this stretch, the elongation terminates and a poly [A] segment is added to the mRNAs by reiterative copying of the stretch of the 'U' residues, by the viral polymerase. The mechanism by which the viral polymerase itself is responsible for the addition of the poly [A] tail to the viral mRNAs is important because of the functions of the viral NS1A protein, which inhibits 3' end processing of cellular mRNAs (reviewed in (Chen and Krug 2000)).

***Replication:***

The replication reaction is carried out in two steps: first, the vRNA is transcribed into full length positive sense *complimentary* RNA (cRNA). The cRNA is then transcribed to give rise to full length negative sense vRNA. Unlike transcription, replication neither depends on a primer for initiation nor is terminated at the stretch of uridines. The NP protein is very crucial for the replication reaction (Shapiro and Krug 1988), (Honda, Ueda et al. 1988), (Medcalf, Poole et al. 1999). NP is encoded by the

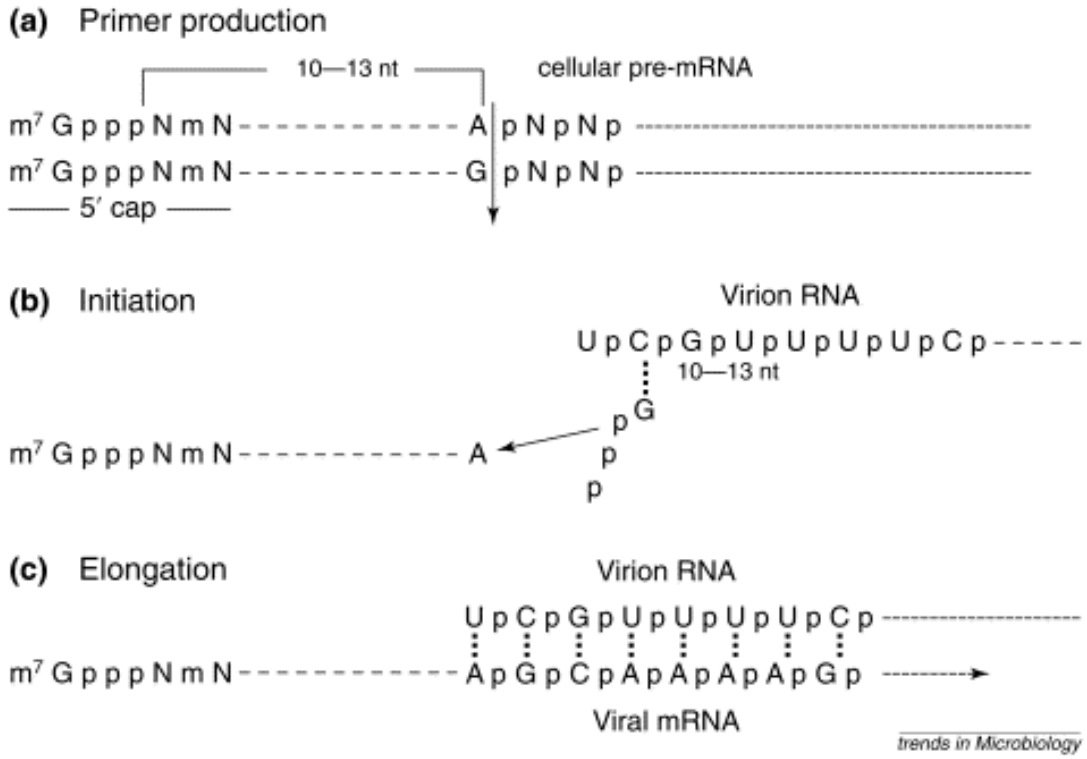



**Figure 1.4** **'Cap snatching' mechanism of influenza A polymerase.** A.Influenza A polymerase contains an intrinsic endonuclease activity which cleaves cellular mRNAs approximately 10-13 nucleotides downstream of 5' end of the RNA. This releases the cap that is then used as primer for transcription of viral messages. B. During initiation, a G is added at the 3' end of the cap structure after which elongation (C) proceeds with the vRNA as template. Adapted from Chen and Krug, 2000.

RNA segment 5 of influenza virus and coats the viral RNA at intervals of 24 nucleotides, thereby covering the entire vRNA. NP is important for 'anti-termination', which prevents the polymerase from terminating at the poly uridine stretch and hence enabling it to transcribe a full length cRNA (Beaton and Krug 1986). NP is also important to mediate the switch from transcription to replication of the polymerase. The RNA binding activity of NP is not required for this switch; rather it has been postulated that the direct binding of NP to the polymerase subunits might be responsible for the 're-modeling' of the polymerase, so that it switches to replication (Newcomb, Kuo et al. 2009).

### *1.3.4 Export of viral RNPs*

The newly synthesized vRNPs must next be exported from the nucleus into the cytoplasm before assembly and budding. The viral proteins necessary for export are M1 and the non structural protein 2 (NS2), also called nuclear export protein (NEP) (O'Neill, Talon et al. 1998). NS2 is encoded from a spliced message of RNA segment 8 of influenza viruses A and B and segment 7 of influenza Virus C. NS2 binds to M1, and export is mediated as a vRNP-M1-NS2 complex through the nuclear pore. For efficient export, there is usually a ternary complex formed between the export substrate, a cellular export factor (CRM1 in case of influenza virus) and Ran-GTP (reviewed in (Boulo, Akarsu et al. 2007)). The nuclear export sequence (NES) on NS2 is essential for the ternary complex formation.

### *1.3.5 Assembly and Budding*

Proper packaging of influenza virus involves the assembly of the eight vRNP complexes contained within the viral envelope. Several recent reports have identified sequences at the 5' and 3' non coding regions as well as some coding sequences of each segment that is important in proper packaging of the virus (de Wit, Spronken et al. 2006), (Dos Santos Afonso, Escriou et al. 2005), (Fujii, Fujii et al. 2005), (Fujii, Goto et al. 2003), (Liang, Huang et al. 2008), (Marsh, Hatami et al. 2007), (Ozawa, Maeda et al. 2009). The integral membrane proteins, HA, NA, (B/C) M2 and NB are synthesized on membrane bound ribosomes and are transported through the endoplasmic reticulum (ER). HA and NA lack the terminal sialic acid on their carbohydrate chains, because of the action of NA, which catalyzes the cleavage of the terminal sialic acid residues on the glycol-proteins. Assembly and budding of the virus takes place at specialized regions of the plasma membrane known as 'lipid rafts', which are enriched in the viral glycoproteins (Scheiffele, Rietveld et al. 1999). The cytoplasmic tails of HA, NA and M2 are crucial in proper assembly of the virus, as they interact with M1, which in turn coats the vRNP forming connections with it. It was recently proved that M1 is not the major driving force for production of virus like particles (VLPs) (Chen, Leser et al. 2007). This is in contrast to several enveloped viruses whose major structural proteins contain a 'late domain' and whose expression alone can drive viral packaging and budding (Bieniasz 2006). At present, host factors involved in influenza virus budding are uncharacterized.

## 1.4 NS1 PROTEIN OF INFLUENZA VIRUSES

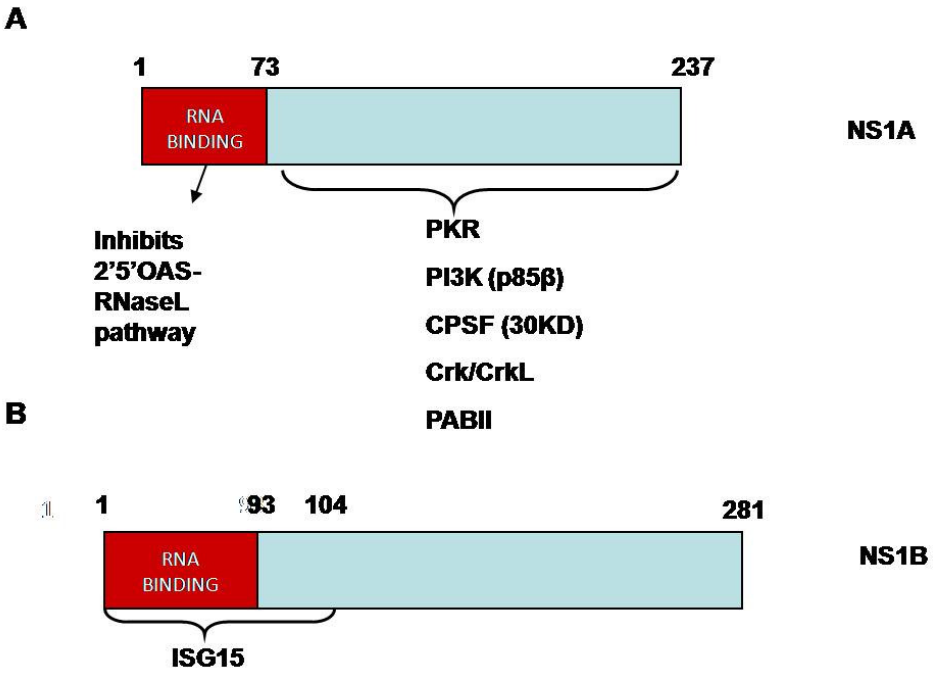



**Figure 1.5 Non structural protein 1 of influenza A and B viruses.** A Schematic representation of NS1A (A) and NS1B (B) showing the N terminal RNA binding domain (red) and C terminal 'effector domain' (blue). Indicated are the known interacting host proteins.

The segment 8 of influenza A and B viruses and Segment 7 of influenza C virus encodes two proteins, named non structural proteins 1 and 2 (NS1 and NS2/NEP). NS1 is encoded by the un-spliced mRNA while the mRNA for NS2 is a spliced version. NS2 functions in nuclear export of the vRNPs (section 1.3.4). Out of all the influenza viral proteins, NS1 and PB2-F2 (of influenza A virus) are expressed exclusively in the infected cell and are absent from the virion. NS1 is a multi-functional protein and has been found to play important roles in viral replication (by regulating polymerase function) (Min, Li et al. 2007), (Falcon, Marion et al. 2004), (Shimizu, Handa et al. 1994), (Scholtissek and Spring 1982) as well as suppressing host innate immune response against the virus (reviewed in (Krug, Yuan et al. 2003), (Hale, Randall et al. 2008)). The critical importance of NS1 is highlighted by the phenotype of recombinant influenza A and B viruses that lack the entire NS1 gene. These viruses are extremely attenuated. While the A/delNS1 virus can be rescued in IFN deficient systems like Vero cells (Garcia-Sastre, Egorov et al. 1998), B/delNS1 virus is highly attenuated both in interferon sufficient and deficient cells (Dauber, Heins et al. 2004). The NS1 proteins of influenza A and B viruses (henceforth referred to as NS1A and NS1B respectively) share similarities in structure and function (Yin, Khan et al. 2007). However the proteins share less than 20% sequence identity, which lead to unique functions for both. These differences in turn highlight differences in viral strategies for combating host defense.

### *1.4.1 Structure and function of the RNA binding domain of NS1A and NS1B*

NS1A and NS1B proteins share an overall similar domain organization: both are

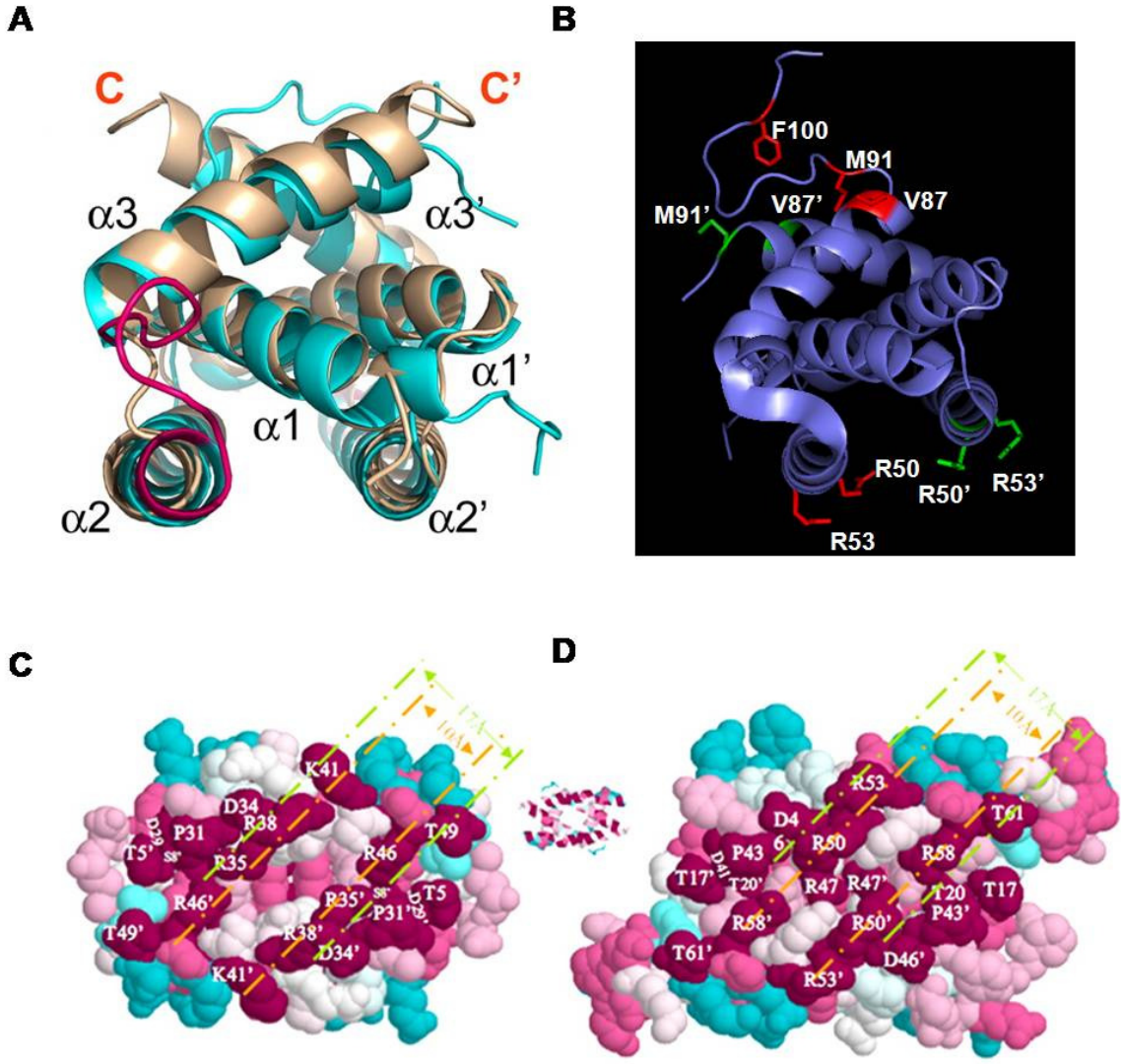



**Figure 1.6 Structure of the dsRNA binding domains of NS1A and NS1B.** A.Overlap of the RNA binding domains of NS1A (tan) and NS1B (blue). NS1B contains a longer loop (red) between helices 2 and 3. B. Dimer of 1-104 amino acids of NS1B with residues important in dsRNA binding (R50 and R53) and those important in ISG15 binding (V87, M91 and F100) highlighted. Red represents residues on one monomer and green represents residues on the other monomer. RNA binding groove of NS1A (C) and NS1B (D) with residues in red representing those showing minimum to no variation. Adapted from (Yin, Khan et al. 2007).

characterized by an N terminal RNA binding domain and C terminal 'effector' domain (figure 1.5). Both proteins are homo-dimers with the N terminal and C terminal both contributing to dimerization. The structure of the RNA binding domain has been resolved by NMR and X ray crystallography for both NS1A and NS1B and found to be highly similar (figure 1.6), (Chien, Tejero et al. 1997), (Liu, Lynch et al. 1997; Yin, Khan et al. 2007), (Cheng, Wong et al. 2009). This domain, which is made up of 1-73 amino acids of NS1A and 1-93 amino acids of NS1B, has a novel six alpha-helical fold formed by a dimer of NS1 with the RNA binding groove made up of positively charged basic residues present in helix 1 and helix 2 of each monomer. The RNA binding domain binds dsRNA in a sequence independent manner, but does not bind dsDNA or RNA/DNA hybrids. The interaction however is weak in comparison to other dsRNA binding proteins and has a dissociation constant ($k_d$) of ~1uM (Chien, Xu et al. 2004). Several residues along the RNA binding groove have been shown to form contacts with the RNA (Yin, Khan et al. 2007); however the critical residue in NS1A is the arginine at position 38 (R38); the mutation of which to alanine completely abrogates dsRNA binding in *in vitro* gel shift assays (Wang, Riedel et al. 1999). By using a recombinant influenza A virus which encoded an NS1 protein with an R38A mutation, the function of dsRNA binding of NS1A in infected cells is to inhibit the 2'5' oligo adenylate synthetase (2'-5' OAS)-RNaseL anti-viral pathway.

There is some controversy regarding the role of the dsRNA binding domain of NS1B. Since the structure of this domain as well as the substrate specificity of NS1B is highly similar to that of NS1A (Wang and Krug 1996), (Yin, Khan et al. 2007), it is reasonable to propose that the function of dsRNA binding in NS1B serves the same

purpose as that of NS1A. However, a group of researchers proposed that the function of dsRNA binding of NS1B was to inhibit the activation of protein kinase R (PKR) (Dauber, Schneider et al. 2006). PKR is an interferon induced, dsRNA activated protein kinase, which is involved in shut down of protein synthesis and apoptosis of the infected cell (reviewed in (Sadler and Williams 2007)). However, the residues in NS1B that were mutated to abolish RNA binding also mis-localize the protein to the cytoplasm (Schneider, Dauber et al. 2009), since this region also forms the sole nuclear localization sequence for NS1B. The absence of a second NLS in NS1B (like in some NS1A proteins) has proved an obstacle to determining the precise role of dsRNA binding of NS1B in infected cells. Also, unlike NS1A which directly binds PKR (Li, Min et al. 2006), the binding of NS1B to PKR is RNA mediated since a R50AR53A double mutant of NS1B that disrupts dsRNA binding (Yuan, Aramini et al. 2002), (chapter 3) also has dramatically reduced PKR binding capacity in co-immunoprecipitation experiments (Sridharan and Krug, unpublished data). However, the R50AR53A mutant NS1B protein is mis-localized to the cytoplasm (chapter 3) and it is not known if NS1B binds to PKR in nucleus or cytoplasm. Therefore, the function of the dsRNA binding domain of NS1B in infected cells is yet to be determined.

### *1.4.2 Structure and function of the 'effector' domain of NS1A*

The C terminus of NS1A constitutes the 'effector' domain and has been shown to be the binding site of several host factors, like the 30kd subunit of the cellular cleavage and poly-adenylation specificity factor (CPSF), poly (A) binding protein II (PABII), PKR and PI3-kinase (PI3K). CPSF30 and PABII are involved in the 3' end processing of

cellular pre-mRNAs that lead to the addition of the poly (A) tail. 3' end processing is a crucial step in the maturation of mRNAs since the inhibition of this step results in unstable messages whose nuclear export is inhibited. NS1A inhibits the activity of CPSF30 and PABII, by binding these proteins (Nemeroff, Barabino et al. 1998), (Chen, Li et al. 1999), thereby preventing the translation of host anti-viral messages (Noah, Twu et al. 2003). The interaction between NS1A and CPSF30 has been well characterized by both structural studies as well as mutational analyses. A crystal structure of the complex of the effector domain of NS1A (amino acids 85-215) and the F2-F3 Zinc finger fragment of CPSF30, which is sufficient to bind NS1A (Twu, Noah et al. 2006), shows that a dimer of NS1A binds two molecules of F2-F3 (Das, Ma et al. 2008). A single point mutation, G184R of NS1A is sufficient to abolish CPSF30 binding and a recombinant influenza A virus whose NS1 contains this mutation is highly attenuated and cannot inhibit the synthesis of IFN message in the infected cell (Das, Ma et al. 2008). Besides G184, two other amino acids outside the binding pocket, F103 and M106, serve to stabilize the NS1A-CPSF30 complex (Twu, Kuo et al. 2007), (Das, Ma et al. 2008), as does the viral polymerase complex which is also present in this complex (Kuo and Krug 2009). The multiple levels at which the NS1A-CPSF30 complex is stabilized underscores its importance in the viral strategy of countering the host antiviral response. The viral messages themselves escape the fate of inhibition of 3' end processing that is mediated by NS1A, since their 3' end processing and poly (A) addition is carried out by iterative copying by the viral polymerase itself and does not depend upon the host machinery (Luo, Luytjes et al. 1991), (Poon, Pritlove et al. 1999), (Robertson, Schubert et al. 1981).

Besides CPSF30, another NS1A interacting host protein is PKR. It has been long observed that PKR activation is inhibited in influenza A virus infected cells and that this inhibition is at least partly mediated by the NS1A protein (Bergmann, Garcia-Sastre et al. 2000), (Hatada, Saito et al. 1999). Recently, it was shown that the mechanism of inhibition by NS1A was by its direct binding to PKR through amino acids 123 and 124 of NS1A, and not due to sequestration of dsRNA away from PKR (Li, Min et al. 2006), (Min, Li et al. 2007).

Recently many studies have shown that NS1A interacts with the p85β but not the p85α subunit of phospatidyl inositol 3 kinase (PI3K) through the amino acids 164-167 as well as amino acids 141 and 142 in NS1A (Hale, Jackson et al. 2006; Shin, Li et al. 2007; Shin, Liu et al. 2007; Li, Anderson et al. 2008); (Hale, Batty et al. 2008). Such an interaction activates PI3K, leading to the phosphorylation and activation of downstream kinase Akt. Studies have also shown that NS1 of avian but not human influenza viruses bind to Crk and/or CrkL via amino acids 212 to 216 (Heikkinen, Kazlauskas et al. 2008), thereby increasing the phosphorylation of Akt. The PI3K-Akt pathway suppresses virus-induced apoptosis, thereby allowing replication of the virus during early times post infection (reviewed in (Ehrhardt and Ludwig 2009)).

### *1.4.3 Functions of the 'effector' domain of NS1B*

Unlike NS1A, NS1B neither binds CPSF30 and inhibits the maturation of host messages (Wang and Krug 1996), nor activates the PI3K-Akt pathway (Ehrhardt, Wolff et al. 2007). Much less is known about the functions of the 'effector' domain of NS1B, because of the lack of knowledge about interacting partners. One characterized

interaction is between NS1B and the cellular protein, the interferon stimulated gene product 15 (ISG15) (Yuan and Krug 2001). ISG15 is an interferon induced 'ubiquitin-like' protein which is discussed in depth in section 1.7. NS1A does not bind ISG15; indeed, in influenza A virus infected cells, little or no ISG15 is made (Kim, Latham et al. 2002) since NS1A blocks 3' end processing of host cell mRNAs. Previous studies in our laboratory showed that the binding site of ISG15 on NS1B is contained within the N terminal 103 amino acids of NS1B (Yuan and Krug 2001). The binding site has contributions from both the dsRNA binding domain (amino acids 1-93) as well as the ten amino acids after the dsRNA binding domain (amino acids 93-103), since neither the dsRNA binding alone nor a NS1B mutant that lacks the dsRNA binding domain but contains amino acids 93-103 is sufficient to bind ISG15. Initially, the amino acids present in loop1 (loop between alpha helices 1 and 2) of NS1B were thought to mediate this interaction (Yuan, Aramini et al. 2002). However, the structure of the dsRNA binding domain of NS1B that was subsequently resolved (Yin, Khan et al. 2007) indicated that these mutations would disrupt the protein structure and was not the true binding site. In the current study (chapter 2), the major binding site for ISG15 on NS1B has been determined and found to involve the third alpha helix of dsRNA binding domain of NS1B. Further, a triple point mutant of NS1B was characterized that disrupts ISG15 interaction but still retains other functions of NS1B like intra-nuclear localization. This suggests that in the triple mutant, the protein structure is mostly retained.

NS1B, like NS1A, inhibits interferon (IFN) β production (Dauber, Heins et al. 2004) although the mechanism of this inhibition must be different from that of NS1A since NS1B does not bind CPSF30. The C terminal effector domain of NS1B is

responsible for this inhibition, as recombinant viruses that contain a truncated NS1B protein of 1-104 amino acids induce dramatically higher IFN β (Donelan, Dauber et al. 2004). Furthermore, unpublished results from our laboratory have shown that the C terminus of NS1B inhibits the activation of the transcription factor, interferon response factor 3 (IRF-3), which induces the transcription of IFN β message. In contrast, several influenza A viruses like Udorn/72 and WSN activate IRF-3 (Kim, Latham et al. 2002), (Das, Ma et al. 2008). It remains to be determined if the inhibition of IRF-3 activation by NS1B accounts for the full inhibition of IFN production.

NS1B shows unique intra-nuclear localization to splicing compartments known as SC35 or splicing speckles, so named for the characteristic accumulation of the splicing factor, SC35 (chapter 3) and (Schneider, Dauber et al. 2009). However, the function for this localization is not understood. SC35 speckles and NS1B's localization to these structures are discussed in detail in section 1.8. NS1B also possesses as yet uncharacterized functions that are not dependent on the interferon response of the cell, since a recombinant virus that lacks the entire NS1 gene is attenuated in both interferon competent MDCK cells as well as in interferon deficient Vero cells (Dauber, Heins et al. 2004). Further studies are necessary to fully understand the role(s) of NS1B in virus infection.

## 1.5 PREVENTION AND TREATMENT OF INFLUENZA

The most effective protection against influenza is vaccination and may include inactivated vaccines (TIV) or live attenuated vaccines (LAIV). The vaccine's

composition is based on currently circulating strains and prediction of future strains for that year and usually consists of a combination of influenza A viruses and an influenza B virus (www.cdc.gov/flu). Vaccination must be performed on a yearly basis because of the variation of circulating strains of influenza between each year. Research is also being done on live attenuated vaccines that mutate critical functions of certain viral proteins which result in a highly attenuated virus (Watanabe, Watanabe et al. 2009), (Wressnigg, Shurygina et al. 2009), (Steel, Lowen et al. 2009).

There also exist anti-viral therapies for influenza, which target the action of various viral proteins. Amantadine (trade name Symmetrel, generic version amantadine hydrochloride) and Rimantadine (trade name Flumadine, generic version rimantadine hydrochloride) are two related anti viral drugs which have two modes of action based on the concentration. Their main mode of action is by inhibiting influenza A viral M2 ion channel protein, which is essential for the virus to un-coat and disassemble after endocytosis. At later stages of the viral infection, these drugs act on the HA molecule (primarily the H7 subtype) to induce a premature pH induced conformational change in HA (summarized from (Lamb and Krug 2001)). Recently, many influenza viruses have developed resistance to Amantadine and Rimantadine (2006 CDC Health alert http://www.cdc.gov/flu/han011406.htm). Another newer set of anti viral drugs, Zamamivir (Relenza®) and Oseltamivir (Tamiflu®), act on the NA protein. NA binds sialic acid, cleaving it from viral and cellular glycoproteins, thus facilitating viral release from the cell. Zanamivir is a sialic acid analog and inhibits the NA of both influenza A and B viruses. Oseltamivir is an orally administered pro-drug of Zamamivir. Current research is also focusing on the potential use of other viral proteins as targets for drugs,

like NS1 and NP proteins. The resolving of crystal structures of these proteins (Yin, Khan et al. 2007), (Bornholdt and Prasad 2006), (Bornholdt and Prasad 2008), (Ye, Krug et al. 2006), along with great advances in the understanding of their functions have identified them to be good targets for the development of molecules that can inhibit their functions.

## 1.6 THE HOST RESPONSE TO VIRAL INFECTION

When a virus infects a host cell, the cell mounts a defense mechanism that ultimately results in what is known as the 'anti-viral state' of the cell. The induction of this state involves hundreds of genes whose action inhibit various stages of viral life cycle like blocking protein translation, preventing virus budding, induction of apoptosis etc. Some of the most important mediators of the host antiviral mechanism are cytokines known as interferons (IFNs). The host response to viral infection takes place in a biphasic pattern; an immediate early response that is independent of IFN and a second late response that is mediated by IFN (reviewed in (Haller, Kochs et al. 2006)). Figure 1.7 describes an overview of the biphasic pattern of host anti-viral response.

### 1.6.1 Early interferon independent anti-viral response

The immediate early IFN independent response does not require viral protein synthesis and results in the induction of several antiviral genes including IFN β (reviewed in (Krug, Yuan et al. 2003), (Haller, Kochs et al. 2006)). This phase is dependent upon the activation of a transcription factor called interferon regulated factor 3 (IRF-3). In recent years, many of the steps in the pathway leading to the activation of IRF-3 have

been described. Upon viral infection of host cell, pathogen associated molecular patterns (PAMPs) like viral genomic ssRNA or dsRNA replication intermediates are recognized by either membrane bound or cellular pattern recognition receptors (PRRs). These two classes of receptors signal through the two distinct pathways described below to activate IRF-3 and subsequently induce transcription of IFN β message (reviewed in (Meylan and Tschopp 2006)).

Membrane bound receptors include the Toll like receptors (TLRs) 3,7 and 8 which are present on the membranes of intracellular organelles (reviewed in (Honda and Taniguchi 2006), (Meylan and Tschopp 2006)). TLR 7 and 8 recognize ssRNA, TLR 3 recognizes dsRNA and TLR 9 recognizes non methylated $C_pG$ DNA motifs (Alexopoulou, Holt et al. 2001), (Diebold, Kaisho et al. 2004), (Heil, Hemmi et al. 2004), (Hemmi, Takeuchi et al. 2000), (Lund, Alexopoulou et al. 2004). Activation of TLRs is achieved by the phosphorylation of their cytoplasmic domains which then signal through various TIR domain containing adaptor proteins like MyD88 or Trif. MyD88 forms a complex with TRAF6 and IRF-7, leading to the activation of IRF-7. Trif recruits the kinase TBK-1 which phosphorylates and activates IRF-3 and IRF-7, ultimately leading to the induction of IFN β message. Trif also leads to activation of the transcription factor NFκB also by recruiting TRAF6 or TRAF3. The TLR pathway for inducing IFN β occurs predominantly in the plamacytoid Dendritic Cells (pDCs), which are specialized cells for producing IFN (reviewed in (Honda and Taniguchi 2006), (Meylan and Tschopp 2006)).

Cellular receptors for viral nucleic acids like RIG-I and MDA-5 are activated by dsRNA, ssRNA or Poly I:C. PolyI:C activates MDA-5 (Kato, Takeuchi et al. 2006), (Gitlin, Barchet et al. 2006) while 5'triphosphate ssRNA was found to be the ligand for

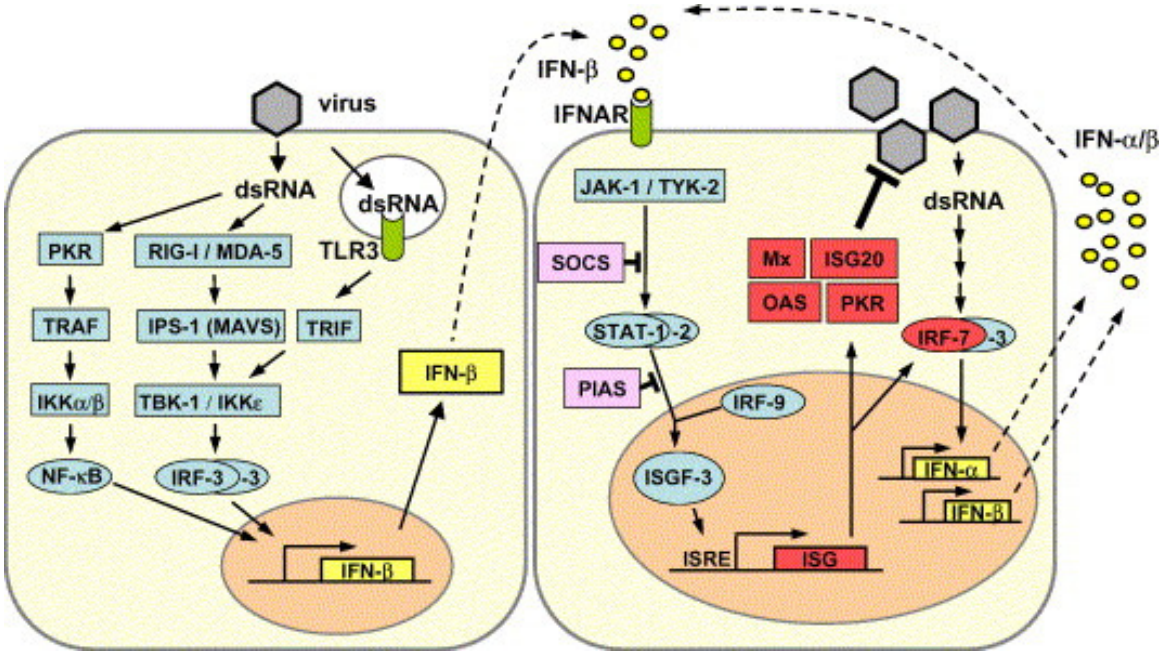



**Figure 1.7** **Biphasic pattern of anti-viral response.** Upon virus infection, signaling by pattern recognition receptors like RIG-I or TLRs result in the induction of IFN β message. IFN β, in an autocrine or paracrine manner, signals through JAK-STAT pathway to induce the full range of IFN induced proteins. Adapted from Haller et al, 2006.

RIG-I but not MDA-5 *in vitro* (Hornung, Ellegast et al. 2006), (Pichlmair, Schulz et al. 2006). Other ligands identified for RIG-I include RNA bearing 5' triphosphate (Hornung, Ellegast et al. 2006), (Pichlmair, Schulz et al. 2006) as well as the poly U/UC part of the 3'UTR of Hepatitis C Virus (HCV) (Saito, Owen et al. 2008). Similarly, influenza A virus infection is able to induce RIG-I activation (Siren, Imaizumi et al. 2006), (Kato, Takeuchi et al. 2006), (Loo, Fornek et al. 2008); however the exact motifs in the viral genome have not yet been discovered. Once activated, RIG-I and MDA-5 signal through the adapter protein variously called MAVS, Cardiff, VISA or IPS-1 (Meylan, Curran et al. 2005; Xu, Wang et al. 2005), (Kawai, Takahashi et al. 2005). Ultimately, this pathway leads to the activation of IRF-3. Different RNA viruses activate IFN β production through either RIG-I or MDA-5 (Kato, Takeuchi et al. 2006), (Loo, Fornek et al. 2008), (Gitlin, Barchet et al. 2006) while some viruses like orthoreovirus, dengue virus and west nile virus signal through both.

IRF-3 is a 55 kDa constitutively expressed protein of the IRF family which to date has 9 members (IRFs 1-9). Signaling through TLRs or RIG-I/MDA5 leads to phosphorylation of IRF-3 in its C terminal domain by the kinases IKKε and TBK-1, resulting in dimerization and activation (reviewed in (Hiscott 2007), (Honda and Taniguchi 2006)). Dimerized IRF-3 translocates to the nucleus where it binds transcriptional co-activators p300 and CREB binding protein (CBP) leading to a virus activated factor (VAF), which binds the promoter element interferon stimulated response element (ISRE) of a subset of ISRE containing genes to induce their transcription (reviewed in (Hiscott 2007), (Honda and Taniguchi 2006)). In a microarray study to identify genes that are directly induced by IRF-3, a subset of interferon stimulated genes

(ISGs) like p54, p56, ISG60 were found to be strongly induced and others like ISG15, 2'5'OAS were modestly induced (Grandvaux, Servant et al. 2002). While IRF-3 alone can activate the above mentioned genes, efficient transcription of IFN β message involves the formation of an 'enhansosome' comprising the transcription factors IRF-3, NFκB, and ATF-2/c-Jun (Du and Maniatis 1992), (Du, Thanos et al. 1993), (Fujita, Miyamoto et al. 1989), (Suhara, Yoneyama et al. 2002). The importance of IRF-3 in the induction of IFN was demonstrated by IRF-$3^{-/-}$ mice which produced much less IFN when infected with encephalomyocarditis virus as compared to wild type mice infected with the same virus (Sato, Suemori et al. 2000). Many influenza A viruses with the exception of A/PR8 virus activate IRF-3 (Kim, Latham et al. 2002), (Das, Ma et al. 2008); however although it was initially shown that influenza B virus activates IRF-3, subsequent experiments both by our lab and others (Donelan, Dauber et al. 2004) have indicated that IRF-3 is inhibited in influenza B virus infection and that this inhibition is mediated by the NS1B protein. The exact mechanism of this inhibition is currently being worked out.

The other important member of the IRF family required for the full induction of interferon response is IRF-7. IRF-7 is considered to be the master regulator because in IRF-$7^{-/-}$ mice, the induction of IFN α is completely inhibited while that of IFN β is greatly reduced (Sato, Suemori et al. 2000). While IRF-3 is a constitutive protein, IRF-7 is virus and dsRNA inducible, except in the case of pDCs, where it is constitutively expressed. Like IRF-3, IRF-7 is activated by phosphorylation in its C terminus. A hetero-dimer of IRF-3 and IRF-7 leads to the full activation of IFN α and all subtypes of IFN β gene. While IRF3 is responsible for the early expression of IFN, IRF7 controls a second late induction of IFN (reviewed in (Hiscott 2007)).

### 1.6.2 Anti-viral response mediated by the interferon system

Interferons (IFNs) are cytokines that were first discovered during influenza virus infection. Interferons play a crucial role in the innate immunity against viruses and other intracellular pathogens. The importance of the interferon system in innate antiviral response of the host is apparent by the phenotype of knockout mice lacking various forms of IFN (Huang, Hendriks et al. 1993), (Muller, Steinhoff et al. 1994), (Hwang, Hertzog et al. 1995) . These mice are unable to effectively counter infection by a wide range of viruses despite having a functional adaptive immune system.

***Classification of Interferons***

There are three main classes of IFNs, classified on the basis of gene sequence, chromosome location and receptor specificity (Pestka, Krause et al. 2004). Type I IFNs consist of fourteen different IFNα, one IFN β, one IFN ω, one IFN Є and one IFN κ proteins. Type I IFNs are produced by all cell types and are recognized by a ubiquitously expressed IFN α Receptor (IFNAR), composed of two chains, IFNAR1 and IFNAR2. Type II IFNs consist of only one IFN, IFN γ. IFN γ is produced only by cells of the immune system like monocytes and lymphocytes and is recognized by the ubiquitously expressed IFN Gamma Receptor (IFNGR), composed of IFNGR1 and IFNGR2 chains. Type III IFNs were recently discovered (Sheppard, Kindsvogel et al. 2003), (Kotenko, Gallagher et al. 2003) and consist of three types: IFN λ1, IFN λ2 and IFN λ3 and are recognized by a receptor comprised of two chains: IFN λR1 which is expressed in specific cell types and the ubiquitously expressed IL-10Rβ.

***Signaling by interferons***

All three types of IFNs follow a similar pattern of signaling (reviewed in (Platanias 2005), (Stark, Kerr et al. 1998)). The binding of ligand to receptor triggers a complex signal transduction pathway involving the receptor associated janus activated kinases (JAKs). Specific JAKs are auto phosphorylated and activated upon engagement of the receptor. The activated JAKs then phosporylate and activate specific latent signal transducer and activator of transcription (STAT) molecules in the cytoplasm. The phosphorylated STATs translocate to the nucleus where they form a complex with other STAT molecules and/or other transactivators to bind to specific elements in the promoters of interferon stimulated genes (ISGs) leading to the transcription of hundreds of genes the products, which set up an 'anti-viral state'. Specific JAKs and STATs that are activated by each type of IFN afford a level of specificity as well as complexity. Type I IFNs activate the JAKs, Tyk1 and JAK1 and STAT1, 2, 3 and 5. While all type I IFNs activate these STATs, IFN α can also sometimes activate STAT4 and STAT5, but these are restricted to certain cell types. The most important transcription activation complex induced by Type I IFNs is the interferon stimulated gene factor 3 (ISGF3) complex. ISGF3 consists of a complex of STAT1, STAT2 and IRF-9. This complex binds to a specific sequence in the promoter of target genes called interferon stimulated response element (ISRE), inducing the transcription of the target genes. IFN γ activates JAK1 and JAK2, which in turn activate STAT1 resulting in the formation of STAT1-STAT1 homodimers which translocate to the nucleus and bind to promoter elements called gamma activated sequences (GAS) to induce the transcription of target genes. Some

genes have only ISRE or GAS while others have both elements; therefore different combinations of STATs can mediate different responses (reviewed in (Platanias 2005), (Stark, Kerr et al. 1998)).

***Interferon induced proteins***

Using microarray analysis of samples from both human and murine cells treated with IFN α, IFN β or IFN γ, over 300 IFN stimulated genes were identified (de Veer, Holko et al. 2001), which expanded a previous study identifying 122 proteins (Der, Zhou et al. 1998). The responsive genes fall into many functional categories, the largest category being host defense or signaling. Some of the IFN induced proteins involved in host defense (PKR, 2'5' Oligo A Synthetase, Mx proteins) have been very well characterized in terms of their mechanism of action and many viruses have strategies for inhibiting or evading them (Levy and Garcia-Sastre 2001), (Haller, Kochs et al. 2006). ISG15 is an ubiquitin-like protein that is induced by type I interferons and also by IRF-3. ISG15 is discussed in detail in the following section since it pertains to my research study.

## 1.7 ISG15

ISG15 belongs to a class of proteins called the 'ubiquitin-like' proteins, which includes SUMO, NEDD8 and FAT10. ISG15 is expressed only in vertebrates and is highly induced upon a variety of stress signals like viral or bacterial infection, interferon treatment, and retinoic acid treatment (Yuan and Krug 2001), (Lenschow, Lai et al. 2007), (Vargas-Inchaustegui, Xin et al. 2008), (Pitha-Rowe, Hassel et al. 2004).

### 1.7.1 Enzymatic cascade and targets of ISG15 conjugation

ISG15 uses a similar enzymatic cascade as ubiquitin to conjugate to target proteins. The cascade for ubiquitin is hierarchical, involving (for vertebrates and sea urchin) two activating enzymes (E1), 10-20 conjugating enzymes (E2s), and hundreds of ligating enzymes (E3s). In an ATP dependent step, E1 activates ubiquitin by forming a thioester bond between a thiol group in its active site and the carboxyl group of the C terminal glycine of ubiquitin. Activated ubiquitin is then transfered to the E2 enzyme, which also forms a similar thiol ester bond involving a thiol group in its active site. The third enzyme in the pathway is the E3. There are two forms of E3s: RING E3s act as scaffolding proteins, bringing the targets in contact with the E2~ubiquitin complex while HECT (homology to E6AP carboxy terminal) domain containing E3s are catalytically active and form a thioester bond with ubiquitin through a thiol group in its active site. Finally, ubiquitin is conjugated to target proteins by formation of an isopeptide bond between the carboxyl group of the C terminal glycine of ubiquitin and the amino group of a lysine in the target (summarized from (Pickart 2001)).

The conjugation cascade for ISG15 is illustrated in figure 1.8. The ISG15 activating enzyme was found to be Ube1L (Ubiquitin E1 like) (Yuan and Krug 2001). Ube1L is deleted in a number of small cell lung carcinoma cell lines (Kok, Hofstra et al. 1993); (McLaughlin, Helfrich et al. 2000); (Pitha-Rowe, Hassel et al. 2004); (Pitha-Rowe, Petty et al. 2004). This observation gave rise to the hypothesis that it might be a tumor suppressor; however direct evidence for its role as a tumor suppressor gene is lacking. Ube1L functions uniquely in the ISG15 pathway, as it cannot activate ubiquitin

(Yuan and Krug 2001). Usually, ubiquitin-like molecules have their own unique E1 enzyme(s) and are unable to utilize ubiqutin E1. This is the case for human and mouse ISG15, however, a recent study of the ISG15 from old world monkeys (owm) like African Green Monkey and the Rheseus Macaque showed that this ISG15 homolog is able to form a thio-ester intermediate with ubiquitin E1 (Pattyn, Verhee et al. 2008). The second enzyme in the pathway, ISG15 conjugating enzyme, was discovered to be UbcH8 (Zhao, Beaudenon et al. 2004). siRNA knock out of UbcH8 almost completely abolishes ISG15 conjugation suggesting that it is the predominant E2 used in ISG15 conjugation. An interesting note is that Ube1L can transfer ISG15 to UbcH8 but not to another closely related E2, UbcH7. This specificity is mediated by structural features contained within the N terminal 39 amino acids of UbcH8, which Ube1L bound specifically (Durfee, Kelley et al. 2008). The last enzyme in the pathway, the ISG15 ligase, is Herc5, which is a HECT domain containing ligase (Dastur, Beaudenon et al. 2006), (Wong, Pung et al. 2006), (Takeuchi, Inoue et al. 2006). Interestingly, knock-down of Herc5 using siRNA in transfection experiments markedly reduced the overall ISG15 conjugation suggesting that Herc5 is the predominant E3 for ISG15 and facilitates ISG15 conjugation to a large number of target proteins. This is unlike the ubiquitin system, where the ligase usually interacts with a specific target(s) and hence mediates target specificity. Herc5 perhaps interacts with adaptor protein(s) that in turn mediate target specificity. Unlike Herc5, another E3, a RING domain containing protein, estrogen-responsive finger protein (EFP), functions as a more specific ISG15 ligase to target the protein 14-3-3σ for ISGylation (Zou and Zhang 2006). The RING domain of EFP is required for the ISG15 conjugation

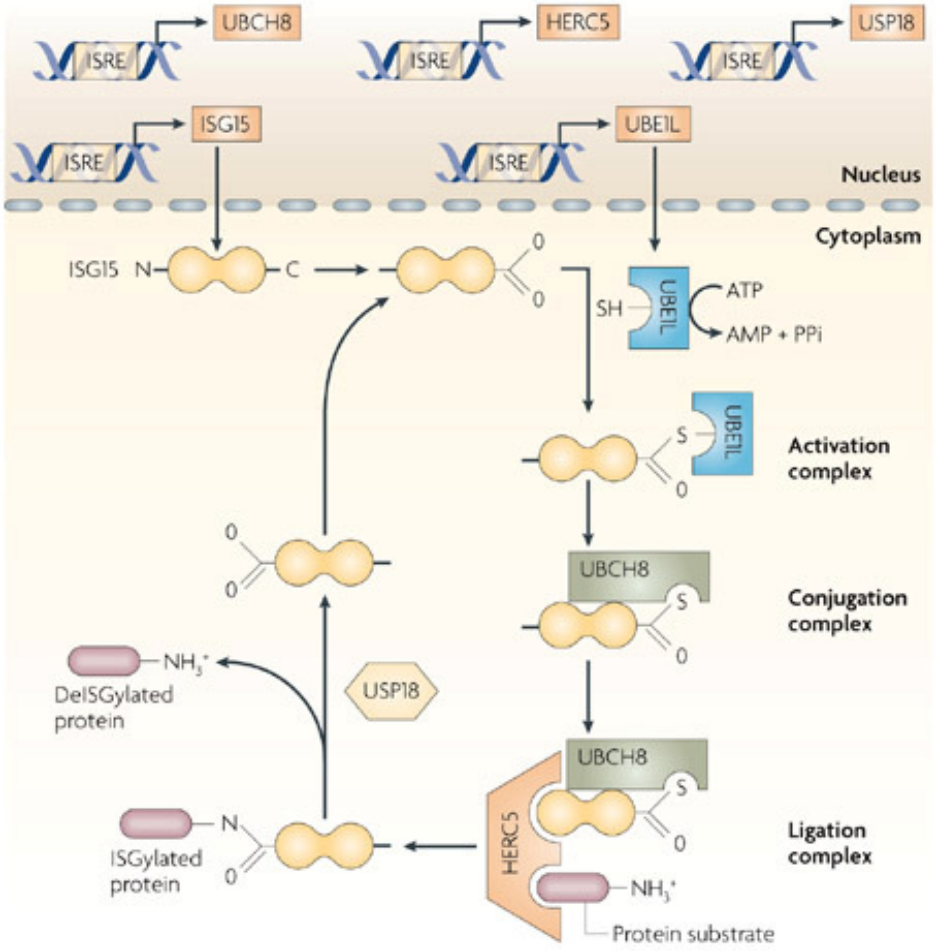



**Figure 1.8 Mechanism of action of ISG15.** See text for details. Adapted from Sadler and Williams, 2008

of 14-3-3σ. Similar to Ube1L, UbcH8, Herc5 and ISG15, EFP is also induced by type I IFNs. Deconjugating enzymes play an important role in the proteolytic processing of precursor ubiquitin and ubiquitin-like proteins to result in the mature form. They also help to recycle ubiquitin and ubiquitin-like proteins by cleaving them off targets to be available to be conjugated to other targets (reviewed in (Wing 2003)). Ubp43 (Usp18) was proposed to be an ISG15 specific protease that removes ISG15 from conjugated proteins (Malakhov, Malakhova et al. 2002). Mice lacking Ubp43 showed elevated levels of ISG15 conjugates and a severe phenotype like brain injury and early death (Malakhova, Yan et al. 2003). However, these effects were not rescued in Ubp43/ISG15 double knock out mice, demonstrating that the phenotype of Ubp43 $^{-/-}$ mice is not related to ISG15 (Knobeloch, Utermohlen et al. 2005). Also, if Ubp43 is the predominant ISG15 processing enzyme, mature ISG15 and its conjugates must be absent in these mice, which is not the case. Therefore, it is unclear what the precise role of Ubp43 is. Like ISG15, Ube1L, UbcH8 and Herc5 are all IFN induced proteins. Therefore, upon IFN treatment, ISG15 is conjugated to a wide variety of targets. During a proteomics study of ISG15 targets in HeLa cells, over 150 targets were discovered, which include proteins involved in a wide range of cellular functions (Zhao, Denison et al. 2005), (Giannakopoulos, Luo et al. 2005). Many of the target proteins are associated with the cytoskeleton. This is consistent with a previous study which found that ISG15 localized to the intermediate filaments (Loeb and Haas 1994). A large number of targets are involved in metabolism and chromatin remodeling/RNA polymerase II transcription. Many of the target proteins are constitutively expressed, but twelve are IFNα/β induced. Out of these twelve, nine

have been shown to have antiviral functions; these include P54, P56, P60, P58, RIG-1, STAT1, MxA, GBP-1 and PKR.

In spite of the identification of many targets, studies on the function of ISGylation of individual proteins have not yielded many conclusive insights. In one study, the mRNA 5' cap binding protein, 4EHP, was found to be conjugated to ISG15 (Okumura, Zou et al. 2007). 4EHP in Drosophila acts as an inhibitor of translation inhibitor (Cho, Poulin et al. 2005), presumably by competing with translation initiation factors, eIF4e and eIF4e3 for binding to the cap. Conjugation of 4EHP to ISG15 led to an enhancement of cap binding activity of 4EHP (Okumura, Zou et al. 2007), however this enhancement was weak. ISGylation also protected IRF-3 from being degraded thereby sustaining and enhancing the IRF-3 response (Lu, Reinert et al. 2006). However another group showed that ISGylation negatively regulates RIG-1 helicase (Kim, Hwang et al. 2008). Since RIG-1 functions upstream of IRF-3 in the same pathway to activate IRF-3, it is unclear why ISG15 conjugation should have the opposite effect on two proteins of the same pathway. Recently, cyclin D1 has been shown to be targeted by ISG15 conjugation, resulting in its inhibition and thus leading to cell growth suppression (Feng, Sekula et al. 2008). Transfection of UBE1L reduces protein stability of cyclin D1, and a lysine-less cyclin D1 is more resistant to UBE1L, suggesting that conjugation to ISG15 is mediating this effect. Similar to other 'ubiquitin-like' modifiers, ISGylated targets constitute a very small (1-5%) fraction of the total target pool in a cell. This has been one of the challenges in attributing function of ISG15 conjugation to the target proteins; the small fraction of conjugated proteins would favor a gain of function rather than a loss of function model for target proteins.

### 1.7.2 Crystal structure of ISG15

The crystal structure of ISG15 has been determined and is shown in figure 1.9 (Narasimhan, Wang et al. 2005). ISG15 has two domains, each characterized by a β grasp fold identical to ubiquitin and separated by a hinge region. Each domain contains a five strand mixed β sheet intercalated by a three turn α helix and also two $3_{10}$ helices that are characteristic of ubiquitin. The authors note that while the orientation of the two domains with respect to each other might represent the most stable solution structure, the hinge region might orient the domains differently when ISG15 interacts with other proteins (Narasimhan, Wang et al. 2005). In its overall organization, ISG15 is very similar to FAT10, which is the only other tandem domain ubiquitin like protein to be identified. The C terminal domain of ISG15 is responsible for forming thiol ester bonds with the activating enzyme UBE1L and the conjugating enzyme UBCH8 (Chang, Yan et al. 2008). The C terminal domain also has a characteristic terminal 'LRLRGG' motif that is conserved among ubiquitin and ubiquitin like proteins and is required for the conjugation to target proteins. The N terminus of ISG15 is predicted to be involved in increasing the number of conjugated targets, since the total number of conjugates of C terminus alone is much lower than that of the full length protein (Chang, Yan et al. 2008). Results presented in this study (chapter 2) and others' (Chang, Yan et al. 2008) show that the N terminus of ISG15 is also important for binding NS1B protein of influenza B virus. My results (chapter 2) also indicate an important role for the hinge region of ISG15 in recognition of NS1B.

### 1.7.3 ISG15 and innate immunity

The observation that ISG15 as well as its conjugating enzymes are all induced by interferon as well as the fact that some of the targets are known antiviral proteins activities has led to the hypothesis that one of the functions of ISG15 conjugation is in the innate host anti-viral response. ISG15 is a secreted protein and functions as a cytokine. It has been implicated in the activation of neutrophils and shown to induce IFNγ from T cells and NK cell proliferation (Owhashi, Taoka et al. 2003), (D'Cunha, Knight et al. 1996), (Recht, Borden et al. 1991). It is not known at this point whether conjugation has any part to play in its function as a cytokine; however an intact C terminal domain containing the 'LRLRGG' motif is required for its biological activity (D'Cunha, Knight et al. 1996).

***Antiviral activity of ISG15***

It has been demonstrated that ISG15 has antiviral activity against specific viruses. The first virus that ISG15 was shown to inhibit was Sindbis virus (Lenschow, Giannakopoulos et al. 2005). Recombinant Sindbis virus expressing ISG15 was shown to be attenuated in mice as compared to Sindbis virus expressing ISG15 that contained a mutation early in its reading frame. Even though the C terminal LRLRGG motif of ISG15 was found to be important for this protection, it is not clear if conjugation of ISG15 per se is required since the protective function of ISG15 was shown in IFN $\alpha/\beta^{-/-}$ mice where conjugation does not occur. ISG15 $^{-/-}$ mice are also more susceptible to infection by influenza A, influenza B, Herpes and Sindbis viruses (Lenschow, Lai et al. 2007). Expression of ISG15 and its conjugates is suppressed in influenza A virus infected cells in tissue culture due to the viral inhibition of 3' end processing of cellular

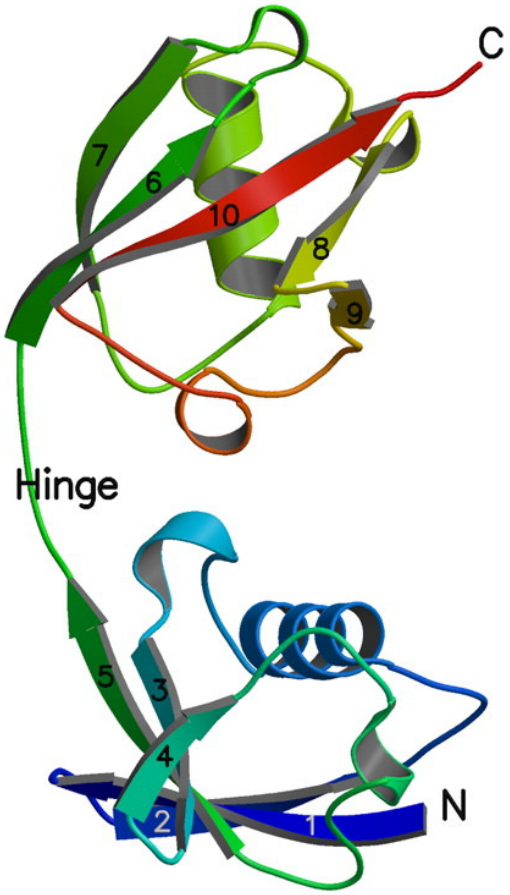



**Figure 1.9 Crystal structure of ISG15.** N and C terminal domains of human ISG15 separated by a hinge are shown. Adapted from Narasimhan et al, 2005.

pre-mRNAs leading to their degradation (Kim, Latham et al. 2002). However, following infection with either influenza A or influenza B virus, wild type mice express free and conjugated ISG15 in the lung. Significantly more ISG15 $^{-/-}$ mice succumbed to influenza A and B virus infection as compared to the control mice (Lenschow, Lai et al. 2007). UBE1L knock out mice showed similar sensitivity to infection with influenza B virus (Lai, Struckhoff et al. 2009). Studies from our laboratory have shown that ISG15 inhibits influenza A virus in human tissue culture (Hsiang Zhao, accepted for publication). When A549 or Calu-3 cells were pre-treated with IFN β before virus infection, wild type influenza A virus was inhibited and rescued upon siRNA knock down of ISG15 conjugation. This rescue was at the level of viral protein synthesis, since a siRNA knock down of another IFN induced protein, Viperin, which inhibits influenza A virus at the budding stage did not affect viral protein synthesis. This is also the first report to conclusively show that conjugation *per se* is antiviral since knock out of UbcH8 alone (which does not deplete the free ISG15 levels) is able to rescue the virus. The mice studies which show that ISG15 has an antiviral activity against influenza B virus (Lenschow, Lai et al. 2007) was surprising to us, since it suggested that NS1B is unable to protect against the anti-viral activity of ISG15 in mice. Indeed, my results (chapter 2) show that that might be the case since NS1B is unable to bind mouse ISG15. My results explain why influenza B virus is rescued in mice lacking ISG15. Recently ISG15 has been shown to inhibit Vaccinia virus replication in vitro (Guerra, Caceres et al. 2008). This study implicated the viral E3 protein to be responsible for inhibiting the activity of ISG15 since an attenuated virus that lacks the entire E3 protein was able to replicate in ISG15 $^{-/-}$ cells but was inhibited in wild type cells. However, it is not clear if the E3

protein is actually responsible for the ISG15 inhibition since transduction of ISG15 in both knock out and wild type MEFs was able to inhibit not only the virus lacking E3 but also the wild type virus. Hence while establishing that ISG15 indeed has an antiviral activity against vaccinia virus, the study does not establish if the virus has any defense against ISG15 that is mediated by the E3 protein. Insights into the mechanism of ISG15's antiviral activity come from studies on HIV and Ebola viruses. Both these viruses are inhibited at the budding stage by ISG15 (Okumura, Lu et al. 2006), (Okumura, Pitha et al. 2008). In HIV life cycle, the ubiquitination of Gag poly-protein and the interaction of Gag with the cellular protein Tsg101 is important for the budding of HIV virions. Ectopic expression of ISG15 and/or UBE1L inhibited the ubiquitination of both Gag and Tsg101 and also inhibits Gag-Tsg101 interaction (Okumura, Lu et al. 2006). siRNA knock down of ISG15 in this study also rescued interferon mediated inhibition of HIV virion budding. Two studies have shown the effect of ISG15 on Ebola virus matrix protein VP40 (Okumura, Pitha et al. 2008), (Malakhova and Zhang 2008). Ubiquitination of VP40 is important for Ebola virus' budding and this ubiquitination is mediated by the ubiquitin ligase Nedd4. ISG15 is able to inhibit Nedd4 mediated ubiquitination of cellular proteins and Nedd4's auto-ubiquitination. One mechanism by which this is accomplished is the binding of ISG15 to Nedd4 in immuno-precipitation experiments which prevents the formation of Nedd4-E2 (UbcH6) complexes (Okumura, Pitha et al. 2008). Furthermore, both these studies show that ISG15 alone is able to inhibit Nedd4 mediated ubiquitination of VP40 both in cell extracts and in VP40 VLPs (virus like particles). This inhibitory effect of ISG15 is mediated by disrupting the interaction of Nedd4 with VP40 through its PTPY late domain since a VP40 that contains a mutated PTPY motif is unaffected by

ISG15. Many other HECT domain containing Nedd4- like E3 proteins are also able to mediate budding of VP40 VLPs and expression of ISG15 alone is able to almost completely inhibit this activity (Malakhova and Zhang 2008).

Some viruses have developed strategies by which they can counter the effects of ISG15. Ovarian tumor (OTU) domain is present on many eukaryotic, bacterial and viral proteases. Some of these proteins have ubiquitin- deconjugating activity. Some RNA viruses like nairoviruses and arteriviruses have been shown to have OTU domain containing proteins. The L protein of the nairovirus, crimean congo hemorrhagic fever virus (CCHFV) has an N terminal OTU domain the expression of which is able to reduce the overall levels of ubiquitinated and IGGylated proteins in a transfection system as well as cleave poly-ubiquitin chains and ISGylated proteins *in vitro* (Frias-Staheli, Giannakopoulos et al. 2007). Transgenic mice expressing the OTU domain of L protein were much more susceptible to infection by a virulent sindbis virus strain, AR86 as compared to the control mice. These results show that viral OTU domains could target the ubiquitin and ISG15 pathways in host innate immune responses. Another viral protein, the SARS coronavirus papain like protease (SARS CoV PLpro), in addition to processing the viral polyprotein, is also a deubiquitinating enzyme that preferentially debranches lysine 48 linked ubiquitin chains (Lindner, Fotouhi-Ardakani et al. 2005). It also preferentially processes ISG15 precursors (Lindner, Lytvyn et al. 2007). The reason why such activity would be of benefit to the virus still needs to be worked out.

ISG15 is not an essential gene as knock out mice show normal development (Osiak, Utermohlen et al. 2005). Moreover, although ISG15 has been shown to have antiviral activity against many viruses, it does not show a universal antiviral role. For

example, ISG15 is not essential for host response against vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) in ISG15 $^{-/-}$ mice. A previous report had suggested that ISG15 has a role to play in STAT1 signaling, as mice lacking a protease (UBP43) that depleted ISG15 conjugates showed increased STAT signaling (Malakhova, Yan et al. 2003). However the ISG15 knock out mice did not show decreased STAT signaling. There was also no increased secretion of IFNγ in the serum of the knock out mice upon VSV and LCMV challenge. These results show that role of ISG15 is critical to protect the host against certain viruses while dispensable against others. ISG15 could play a broader role in the host's innate response to infection not only to viruses but also to intracellular bacteria. Recently, it has been shown that *Leishmania braziliensis* infection induces ISG15 (Vargas-Inchaustegui, Xin et al. 2008), as does bacterial lipopolysaccharide (LPS).

Influenza B virus is currently the only known virus that encodes a protein that directly binds ISG15 (Yuan and Krug 2001). NS1B reduces the intracellular levels of ISG15 conjugates in transfection experiments (chapter 2). Hence, it is hypothesized that ISG15 (and/or) conjugation plays an important anti-viral role in the life cycle of influenza B virus and NS1B inhibits these activities. While many of the previously mentioned studies on ISG15 use ectopic over expression of ISG15 to study its role in antiviral activity (Okumura, Pitha et al. 2008), (Okumura, Lu et al. 2006), (Lenschow, Giannakopoulos et al. 2005), (Guerra, Caceres et al. 2008), influenza B virus offers a unique possibility of making a mutant virus in which the NS1B is unable to bind ISG15. Such a virus would afford a system where the role of ISG15 can be studied in tissue culture without any over expression of ISG15 and therefore will be physiologically relevant. Such a mutant virus

would be predicted to be attenuated as compared to the wild type and would be able to provide insights into the mode of action of ISG15. In the current study, the ISG15 binding site was determined and a mutant virus generated whose NS1B contained a mutated ISG15 binding site. The characterization of this mutant virus is the one of the major focuses of my dissertation.

## 1.8 INTRA NUCLEAR COMPARTMENTS AND SC35 SPECKLES

The nucleus is a highly compartmentalized structure, with several components (both nucleic acids and proteins) organized into distinct sub nuclear compartments, like nucleolus, PML bodies, cajal bodies, gems, para speckles, and SC35 speckles (Spector 1993), (Lamond and Spector 2003). These compartments show punctuate distribution in microscopy and are usually identified by microscopy as well as the presence of characteristic proteins.

SC35 speckles or splicing speckles are named for the characteristic accumulation of the splicing factor SC35 in these structures. Each cell contains 20-50 SC35 speckles depending on the stage of the cell cycle and the transcription state of the cell. In electron microscopy, speckles are divided into two distinct domains: 'core' inter chromatin granules (ICGs), each 20-25nm in diameter, and 'fibrilar extensions' at the edges of the ICGs called perichromatin fibrils (PFs). Besides the periphery of the speckles, PFs are also found at other regions of the nucleus, far away from the speckles (reviewed in (Lamond and Spector 2003)).

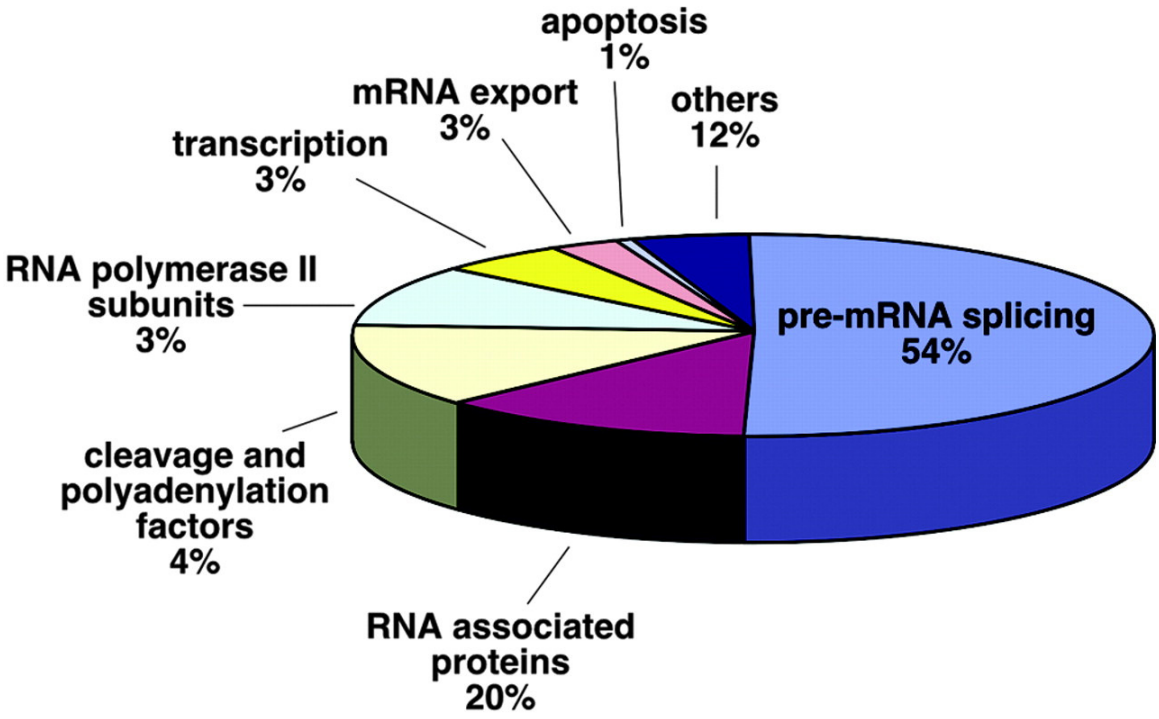



**Figure 1.10 Protein composition of splicing (SC35) speckles.** Pie chart showing the distribution of protein classes contained in mouse liver speckles. One hundred forty six proteins identified in speckles are classified based on their proposed functions. Adapted from Saitoh, Spahr et al. 2004.

### *1.8.1 Composition of speckles*

The protein composition of the speckle has been identified using a biochemical purification of the nuclear speckles from mice liver followed by mass spectrometry (figure 1.10) (Mintz, Patterson et al. 1999), (Saitoh, Spahr et al. 2004). In these studies, 146 proteins were identified out of which 81% were involved in RNA metabolism. This included proteins involved in transcription (RNA polymerase II subunits), mRNA export, RNA binding proteins and pre-mRNA splicing, which comprised of over 50% of the identified proteins. The splicing factors SR proteins are especially enriched in speckles. SR proteins are a conserved family of proteins involved in a wide variety of activities in both constitutive and regulated splicing (Zahler, Lane et al. 1992), (reviewed in (Fu 1995), (Valcarcel and Green 1996), (Kramer 1996)), and are characterized by the presence of one or two RNA recognition motifs (RRMs) and a RS domain, which contains many tens or hundreds of repeats of the dipeptide, arginine-serine. The RS domain has been proposed to play a role in protein-protein interactions, and is also heavily phosphorylated at the serine residues present in this domain (reviewed in (Stamm 2008)). Besides proteins, speckles also contain a stable pool of poly A+ RNA (Carter, Taneja et al. 1991), (Visa, Puvion-Dutilleul et al. 1993), (Huang, Deerinck et al. 1994). This RNA population is not exported into the cytoplasm (Huang, Deerinck et al. 1994); therefore it is proposed that these either play a structural role in the maintenance of the speckles or represent defective mRNAs. The function of this RNA population in the speckles is still under study.

Analysis of the proteins identified in the speckle did not reveal any single common motif that could target them to these speckles. Indeed, several different speckle

targeting domains have been described. One of the well studied domain is the RS domain of SR proteins. Some SR proteins like SC35 and SRp20 contain a single RNA recognition motif (RRM) and RS domain. For these proteins, the RS domain is important for speckle localization (Caceres, Misteli et al. 1997). The RS domain is also necessary and sufficient for speckle localization of Drosophila proteins like Tra and SWAP which do not contain a RRM (Li and Bingham 1991; Hedley, Amrein et al. 1995). Some SR proteins like SF2/ASF and SRp40 contain not one but two RRMs. In these proteins, the RS domain is not required for speckle localization *per se* but might be involved in nuclear targeting (Caceres, Misteli et al. 1997). In these proteins, any two of the three domains are required for speckle localization. Besides the RS domain, other speckle localization signals have been described. The polyhistidine tail of the kinase DYRK1A (Alvarez, Estivill et al. 2003), two contiguous sequences rich in arginine, proline and serine in the SR related protein SRm160 (Wagner, Chiosea et al. 2003), the ankyrin repeat of IκBL protein (Semple, Brown et al. 2002) and the fork-head domain of protein phosphatase I regulator NIPP1 (Jagiello, Van Eynde et al. 2000) have all been shown to act as speckle targeting motifs in those respective proteins. For some proteins, the nuclear and speckle localization signals can be separated, but for others, these seem to be inseparable. In summary, it can be concluded that the signals mediating speckle localization vary widely between different proteins.

### *1.8.2 Model of speckle function*

The exact function of the speckles has been the subject of debate. One model suggests that the speckle plays an active role in mRNA processing and screening for

defective mRNA before it is exported to the cytoplasm (reviewed in (Hall, Smith et al. 2006)). The other more commonly accepted model proposes that the speckles act more like sites of storage or assembly for splicing and transcription factors from where they are recruited to the perichromatin fibrils, which are sites of active transcription and RNA processing (Jimenez-Garcia and Spector 1993). Indeed, speckles are dynamic structures and although the position of the speckles themselves remained constant in the nucleus, there is a constant movement of factors to and from the speckles in inter-phase nuclei. Time lapse microscopy of GFP tagged splicing factor SF2/ASF showed movement at the periphery of the speckles (Misteli, Caceres et al. 1997). Several conditions regulate the movement of factors to and from the speckles as well as their morphology. The movement of GFP tagged SF2/ASF did not occur when the cells were treated with α amanitin, a RNA polymerase II inhibitor (Misteli, Caceres et al. 1997). Conversely, in virus infected cells in which transcription occurs at high rates, the speckles disperse and lose their compact rounded structures (Bridge, Xia et al. 1995). The status of phosphorylation of the SR proteins also affects speckle assembly. The serines in the SR domain of these proteins are phosphorylated by kinases like SRPK-1 (Gui, Lane et al. 1994) and CLK/SKY (Colwill, Pawson et al. 1996). Phosphorylation regulates the association of SR proteins in the spliceosome (reviewed in (Stamm 2008)). When SRPK-1 was added to permeabilized cells or CLK/SKY was over expressed, the splicing factors were released from the speckles into the nucleoplasm, while protein phosphatase-1, which dephosphorylates SR proteins, causes enlargement and rounding of speckles (Misteli and Spector 1996). Similarly, inhibition of splicing by microinjection of oligo-nucleotides or antibodies into cells also cause the speckles to enlarge, become fewer in

number and round up, suggesting an accumulation of splicing factors in these bodies (O'Keefe, Mayeda et al. 1994).

### *1.8.3 NS1B and SC35 speckles*

It has been observed in my research (chapter 3) and others' (Schneider, Dauber et al. 2009) that NS1B localizes to SC35 speckles. At the time of initiation of this study, the speckle localization of NS1B was not characterized or previously reported. NS1B localizes to the speckles at early times post infection as well as in transfection experiments, indicating that other viral proteins are not required for this activity. Also, N terminal tagging of NS1B with V5 or GFP did not affect the localization (chapter 3). Subsequently, a paper was published which described the localization of NS1B to speckles (Schneider, Dauber et al. 2009). Both my data as well as this study show that at least part of the localization sequence is found within the N terminal 93 amino acids of the protein. However, this study failed to identify a specific sequence necessary for speckle localization that was distinct from the nuclear localization signal (NLS). In this study (chapter 3), I show that speckle localization signal is different from the NLS and is contributed by both N and C terminus of NS1B. The reason for NS1B's localization is intriguing. NS1A which binds PABII(a speckle localized protein), is not localized to speckles. Rather, NS1A draws out PABII from speckles to the nucleoplasm (Chen, Li et al. 1999). NS1B does not bind PABII or CPSF30 and does not inhibit 3' end processing. NS1B also has no characterized effect on the host splicing machinery (Wang and Krug 1996). Therefore the function of this sub-nuclear compartmentalization of NS1B is not

known. The current study was initiated to further characterize the localization of NS1B; in particular, identify regions of the protein that could act as the targeting signals.

Relatively little is known about the strategies used by influenza B virus for its replication and suppression of host response. My research has focused on the characterization of NS1B protein of influenza B virus in an effort to better understand the functions of this protein in virus infection. In particular, I have focused on two properties of the NS1B protein that are not shared by its counterpart in influenza A viruses, NS1A, namely, binding of NS1B to the host factor ISG15 (chapter 2) and its unique intra-nuclear localization to splicing speckles (chapter 3). By characterizing these two functions, my research aims to shed further light into the strategies adopted by influenza B virus for its replication and in combating host immune response. One of the goals of my research was study the importance of NS1B-ISG15 interaction during influenza B virus infection using a recombinant virus which encodes an NS1B cannot bind ISG15. Another goal of my study was to further characterize the intra-nuclear localization of NS1B to nuclear speckles.

# CHAPTER 2: Influenza B virus NS1 protein exhibits species specific interaction with ISG15

## 2.1 INTRODUCTION

Interferons (IFNs) form a crucial component of the innate immune response to viruses and other intra-cellular pathogens. Upon IFN induction, hundreds of genes are induced whose products are involved in inhibiting various stages of the viral life cycle or inducing host cell apoptosis (de Veer, Holko et al. 2001). Interferon stimulated gene 15 (ISG15) is one of the earliest and most strongly induced genes by type I interferons (IFN α/β). Upon IFN induction, ISG15 is conjugated to hundreds of intracellular proteins through an enzymatic cascade involving an E1 activating enzyme, E2 conjugating enzyme and E3 ligating enzyme (figure 1.8, reviewed in (Sadler and Williams 2008)). It is first attached to the E1 activating enzyme (UBE1L) (Yuan and Krug 2001) through a thioester bond in its active site, from where it is transferred to the conjugating enzyme (UBCH8) (Zhao, Beaudenon et al. 2004) again forming a thioester bond mediated complex with the E2. Finally it is conjugated to target proteins via an E3 ligase (HERC5) (Dastur, Beaudenon et al. 2006), (Wong, Pung et al. 2006). ISG15 and its conjugation have been shown to have anti-viral activities against several viruses (Lenschow, Lai et al. 2007), (Okumura, Lu et al. 2006), (Okumura, Pitha et al. 2008), (Guerra, Caceres et al. 2008), (Lai, Struckhoff et al. 2009). In many of these cases, ISG15 alone can inhibit the viruses, with the exception of influenza A virus, where it was shown in our laboratory

that *conjugated* and not free ISG15 is important for the inhibition (Hsiang, Zhao accepted for publication).

The non structural protein 1 of influenza B viruses (NS1B) plays an important role in the viral life cycle since a deletion of it from the virus dramatically attenuates the virus in both interferon competent as well as deficient cells (Dauber, Heins et al. 2004). NS1B shares a similar N terminal dsRNA binding domain as its counterpart in influenza A viruses (NS1A) (Wang and Krug 1996), (Yin, Khan et al. 2007). However this region also contains certain unique features that are not conserved between the two proteins. One such feature is the binding of NS1B but not NS1A to ISG15 (Yuan and Krug 2001). The region of NS1B that is necessary for this binding is contained within the N terminal 103 amino acids. Using GST binding assays, it was determined that part of the binding site on NS1B was contributed by the 1-93 amino acids that constitute the dsRNA binding domain and the other part of the binding site was contributed by amino acids between 93 and 103 (Yuan and Krug 2001). The binding to ISG15 and dsRNA by NS1B are independent of each other, and can occur simultaneously resulting in a super complex (Yuan, Aramini et al. 2002). Initially, it was proposed that loop1 of NS1B (the loop between alpha helices 1 and 2) was involved in ISG15 binding since mutations in this loop disrupted binding. However, this mutant was based on a structure prediction for N terminus of NS1B (Yuan, Aramini et al. 2002), which has been subsequently proven wrong (Yin, Khan et al. 2007).

In this study, we identified the correct binding site of ISG15 on NS1B. We identified a role for helix 3 in the RNA binding domain of NS1B in the binding of ISG15. Further, we identified a residue between amino acids 93 and 104 also involved in

binding, thus confirming the previous study that predicted a role for both these two regions in binding ISG15. We introduced this mutation into the virus by reverse genetics to generate a recombinant B/Yamanashi/98 virus whose NS1B cannot bind ISG15. We show that the wild type NS1B is able to sequester ISG15 into intra nuclear speckles in infected cells while the mutant NS1B is unable to do so. Furthermore, we determined the species specific nature of the interaction between ISG15 and NS1B by showing that NS1B binds to ISG15 from humans and old world monkeys, but not to mouse or canine ISG15. Consistent with this data, a recombinant Influenza B virus which encodes a NS1B protein that does not bind ISG15 is attenuated in human cells but not in canine cells. We also identified an important role for the hinge region between the N and C terminal 'ubiquitin-like' domains of ISG15 in determining species specificity. Our results have highlighted the importance of using human tissue culture systems in studying the role of host factors in influenza B virus infection.

## 2.2 MATERIALS AND METHODS

### *Cell lines and plasmids*

Madin-Darby canine kidney cells (MDCK) (ATCC CCL-34), African green monkey Cos7 (ATCC CRL-1651), human lung carcinoma cells A549 (ATCC CCL-185) and Calu-3 (ATCC HTB-55) were purchased from ATCC. Hela tet-on cells were purchased from Clonetech (catalog # 630901). MDCK, Cos7, 293T, A549 and Hela tet-on cells were cultured in DMEM (GIBCO ®) supplemented with 10% heat inactivated fetal bovine serum (FBS) (GIBCO ®), and 2mM 1- glutamine, 100units/ml penicillin and

100μg/ml streptomycin (GIBCO ®) at 37°C with a 5%$CO_2$/95% air atmosphere. Calu-3 cells were cultured in Advanced MEM (GIBCO ®) supplemented with 10% heat inactivated fetal bovine serum (FBS) (GIBCO ®), and 2mM 1- glutamine, 100units/ml penicillin and 100μg/ml streptomycin (GIBCO ®) at 37°C with a 5%$CO_2$/95% air atmosphere.

***Expression of GST tagged proteins in bacteria and their large scale purification by glutathione sepharose affinity selection***

For expression of proteins for *in vitro* ISG15 binding assay, the DNA for NS1B wild type or mutant (AAA) full length was cloned into expression vector pGEX4T2 (Amersham) using the sites BamH1 and Xho1 on the vector and 5' BglII and 3' XhoI for the insert. The enzyme sites were introduced into the ends of NS1B DNA by PCR based mutagenesis. For *in vitro* thio-ester assays, DNA for NS1B full length was cloned into pGEX3X vector (Amersham) using BamH1 site on the vector and BglII on insert. DNA for ISG15 was cloned into pGEX4KT plasmid and pGEX-UBCH8 plasmid was obtained from Dr. Brenda Schulmann (St. Jude's Children's hospital, Memphis, Tennessee).

Single colonies of *E.coli* DH5α strain transformed with the above plasmids were grown in 1litre of LB containing 100ug/ml ampicillin. Protein expression was induced by 0.1M IPTG (isopropyl thio galactoside) for 3-4 hours at 37ºC. Cells were pelleted, washed and re-suspended in 15ml of ice cold PBS along with Complete EDTA free protease inhibitor (Roche). Cells were lysed using sonication, and addition of 1.5 ml of 10% triton X 100 detergent. The cell debris was pelleted by centrifuging the samples at 13,000 rpm for 45 minutes in Beckman centrifuge. The supernatant was added to a

prepared column with a 1ml bed volume of glutathione sepharose beads (GE Healthcare). After washes with PBS and PBS/0.1% triton X 100, the bound proteins were eluted with glutathione elution buffer (10mM glutathione and 50mM Tris pH8.0). Protein purity and concentration were confirmed by SDS gel analysis and Bradford's assay.

***In vitro GST binding assay***

Bacterially purified GST NS1B wild type or AAA mutant protein (5µg) was bound to 30µg glutathione sepharose beads (GE Healthcare) for 2hr at 4ºC along with 200µl of extracts from Hela cell that were treated with 1000units of human IFNβ for 40h (extracts were prepared in 1% NP40 containing lysis buffer) in the presence of buffer containing 25mM Tris-Cl pH8.0, 50mM NaCl and protease inhibitors. Beads were then washed with T25N50 buffer containing 0.1% NP40 before being boiled. Bound proteins were separated by 12% SDS PAGE and subjected to immunoblotting with rabbit anti NS1B or rabbit anti ISG15 (raised against GST ISG15 by Cocalico) antibodies.

***GST binding assay from transfected 293T cell extract***

293T cells in 6 well plates were transfected with plasmids encoding NS1B and GST tagged ISG15 wild type or mutants (generated by PCR based mutagenesis) that were cloned into the PCN vector. 24-48h post transfection, cell lysates were prepared in 200µl of lysis buffer (50mM Tris pH7.5, 150mM NaCl, 5mM EDTA, 2.5mM $MgCl_2$, 1% NP40, 10% glycerol and protease inhibitor). Cell lysate (100-120µl) was mixed with equal volume of lysate dilution buffer (50mM Tris pH 7.5 and 150mM NaCl) and added to 50-60µl of glutathione sepharose beads (GE Healthcare) and incubated on a rotator in

4ºC for 3-4h. Beads were then washed with wash buffer (20mM Tris pH 7.5, 150mM NaCl and 0.5% NP40) three times before being boiled. Proteins bound to beads were separated by 2% SDS PAGE and detected by immunoblotting with anti NS1B or anti GST antibodies to detect NS1B and GST fused to ISG15 respectively.

***Generation and amplification of recombinant influenza B/Yamanashi/1998 viruses***

The desired mutation (AAA) in NS1 gene was generated by PCR based mutagenesis and cloned into the Bsmb1/Esp3I sites of a pCDNA3 based pAD3000 plasmid vector. Recombinant viruses were developed using the eight- plasmid based reverse genetics system described previously (Hoffmann, Mahmood et al. 2002). Briefly, cos7 cells were co-cultured with MDCK cells in six well plates and co-transfected with eight pAD3000 plasmids, each having one genomic segment of the virus cloned into it. Each viral segment is flanked by a RNA polymerase I and RNA polymerase II promoter. Inside the cell, transcription from the RNAPII promoter would give rise to positive sense capped and poly-adenylated mRNAs which are translated into viral proteins. Transcription from the RNAPI promoter gives rise to full length negative strand viral RNA segments which together with the viral proteins form vRNP complexes. Viruses are budded off from the surface of the cos7 cells and harvested from the supernatant. When co-cultured with MDCK cells, virus released from cos7 cells is amplified in MDCK cells to yield higher titer.

The N terminal 3xflag wild type NS virus was generated by Park, J.W in our laboratory (unpublished data). Following a similar strategy, a 3Xflag sequence was introduced into the N terminus of AAA mutant NS gene by a two step PCR, in which the

first PCR used a 5' primer that partially overlapped with the 5'primer used in the second PCR, together generating the full sequence of the 3X flag tag. The final PCR product was digested with Bsmb1/Esp3I and cloned into a similarly digested pAD3000 plasmid. This plasmid was then used in the reverse genetics system to generate the 3Xflag tagged AAA virus as described above.

Viruses were amplified by injecting 100µl of cell supernatant from reverse genetics experiment (diluted in serum free DMEM) into the allantoic cavity of ten day old embryonated chicken eggs, followed by incubation at 33ºC for 2 days. For virus amplification in MDCK cells, the cells were infected with dilutions of virus in serum free DMEM and allowed to adsorb for 1hr at 34ºC after which the cells were washed and incubated with opti-MEM containing 2.5µg/ml N acetyl trypsin (NAT) and 1% bovine serum albumin (BSA). Influenza B wild type and AAA mutant viruses were amplified in ten day old embryonated eggs. 3xflag wild type and AAA mutant viruses were amplified in MDCK cells.

For virus sequencing, the viral RNA was extracted from egg or MDCK amplified virus using Qiagen viral RNA extraction kit. The vRNA was then used as template in reverse-trancription (RT) reaction using Roche RT kit and random hexamers as primers to generate complementary strand RNA. The NS (or other viral genes) were amplified using PCR and segment specific primers. The sequence was confirmed by sequencing of the PCR product.

***Virus infections***

For multiple cycle infections, MDCK or Calu-3 cells were grown to 80-90% confluency in 60mm dishes, washed with PBS, and incubated with virus diluted in serum free DMEM, at an multiplicity of infection (moi) of 0.001. After 1hr of adsorption at 34ºC, the innoculum was removed, cells were washed with PBS and over-layed with opti-MEM supplemented with 1% penicillin streptomycin glutamate (PSG) along with 2.5ug/ml N acetyl trypsin (NAT). At specific time points, the supernatants were taken for titer determination by plaque assay. To assay for the requirement of NAT in multiple cycle growth in calu-3, the incubation media lacked NAT or NAT of 1 or 2.5 ug/ml. For multiple cycle infections of Hela tet-on and A549 cells, these cells were grown on collagen coated 60mm dishes (for collagen coating, 2-3ml of 0.01% weight/volume of rat tail collagen I (Sigma; stock concentration 0.1% weight/volume in 0.1M acetic acid) in PBS was added to dishes, and let sit for 2-3hr in the 37ºC incubator, after which the collagen was removed and plates allowed to dry before plating cells), and infected with virus at a moi of 0.01. After adsorption, the cells were washed and over layed with opti-MEM supplemented with 1µg/ml NAT. 0.5µg/ml NAT was replenished every 36hr.

For single cycle infections, cells were infected with virus diluted in serum free DMEM, at a moi of 2-5. After adsorption, the cells were washed three times with PBS, and over-layed with opti MEM containing 1% PSG. At specific times post infection, the cells were processed for Western blot or immunofluroscence analysis.

***Viral titer determination by plaque assay***

MDCK cells were grown to confluency on 60mm dishes. Prior to infection, the media was removed and cells washed with PBS. Dilutions of influenza B virus wild type

or AAA mutant (in serum free DMEM) were added to the cells and allowed to adsorb for 1hr at 34ºC. Cells were washed with PBS, and over-layed with serum free DMEM containing a final concentration of 2.5ug/ml N acetyl trypsin and 1% agarose. Once the agarose solidified, the plates were inverted and incubated at 34ºC for 3-5 days until plaques start to appear. At the time of staining, the agarose was removed and the cells stained with Naphthalene blue-black stain (1g Napthalene blue black powder, 60ml glacial acetic acid, 13.6g anhydrous sodium acetate in 1 liter distilled water) for 10min at room temperature to count the plaques. The viral titer is calculated by the following formula:

Viral titer = # of plaques*dilution factor/ volume of innoculum

***Immunofluroscence and confocal microscopy***

Hela tet-on cells were grown in four chamber cover glass bottom microscopic slide (Lab Tek) and transfected or infected as desired. At the desired time, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. Following three washes with PBS, the cells were permeabilized in 0.5% tween 20 in PBS for 10 minutes on ice. Cells were then washed three times with PBS and incubated in blocking solution (PBS-NGS-gelatin; 0.5ml of 100% normal goat serum and 0.2ml of 10% gelatin in 1X PBS) for half an hour at room temperature followed by incubation with primary antibody (1:200 dilution in PBS-NGS-gelatin blocking solution) for one hour at room temperature or overnight at 4ºC. Primary antibodies used included rabbit anti NS1B antiserum (raised against GST NS1B), mouse anti SC35 monoclonal antibody (Sigma), mouse anti flag M2 monoclonal antibody (Sigma) and ferret anti

influenza B/Memphis antiserum (kindly provided by Dr. McCullers, St. Jude's Children's hospital, Memphis, Tennessee). Following incubation with primary antibody, the cells were washed 3 times with PBST (PBS with 0.1% tween 20), and incubated with secondary antibody (1:200 dilution in blocking buffer) in dark for 30 minutes at room temperature. Secondary antibodies used included goat anti rabbit IgG conjugated to TRITC (Jackson Labs), goat anti mouse IgG that was TRITC (Jackson Labs) or FITC (Sigma) conjugated and goat anti ferret IgG that was FITC conjugated (Bethyl laboratories). After incubation with secondary antibody, the cells were washed as described above (in dim light), stained with DAPI (1µl of 1mg/ml stock concentration in 15ml PBS) in dim light, washed with PBS and then observed using the 40X oil immersion objective in the Leica confocal microscope at the U.T Austin ICMB core facility. The images were processed by LCS Lite software.

***Transfections***

Transfections were carried out using Mirus transfection reagent according to the manufacturer's protocol.

***Immunoblotting and antibodies***

Proteins were resolved on 10-12% SDS PAGE using the Biorad mini-gel system, and transferred to a nitrocellulose membrane using semi-dry Western transfer apparatus using 0.8mA/cm$^2$ current. For immunoblotting, membranes were blocked with TBST (Tris Borate Saline containing 0.2% tween 20) containing 5% milk for 30 minutes at room temperature, followed by incubation with primary antibody diluted in blocking

buffer for 1-2h at room temperature or overnight at 4ºC. Primary antibodies used included rabbit anti GST NS1B antiserum (1:1500 dilution), goat anti GST antibody (1:10,000 dilution), rabbit anti ISG15 antiserum (1:1000 dilution), rabbit anti P56 antiserum (1:1000 dilution), mouse anti flag M2 monoclonal antibody (Sigma, 1:1000 dilution) and mouse anti tubulin monoclonal antibody (1:1000 dilution). Following incubation with primary antibodies, the membrane was washed three times with 15-20ml of TBST for 10 minutes each at room temperature, and then incubated with secondary antibody diluted in blocking buffer for 1h at room temperature. Secondary antibodies used included goat anti rabbit IgG conjugated to HRP (Zymed, 1:10,000 dilution), goat anti mouse IgG conjugated to HRP (Zymed, 1:10,000 dilution), or donkey anti goat IgG conjugated to HRP (Santa Cruz, 1:10,000 dilution). Following incubation with secondary antibody, the membrane was washed with TBST as described above and developed with Pierce ECL chemiluminscent substrate. For ISG15 westerns, the blot was developed using Pierce supersignal west pico chemiluminescent substrate.

***In vitro assay to detect ISG15 thiol ester bond formation with UBE1L and UBCH8***

For preparation of $^{32}P$ labeled ISG15, 5µg of bacterially expressed GST ISG15 protein was bound to glutathione beads for 2 hours on a rotator at 4ºC. Beads were washed with PBS and kinase buffer (40mM Tris pH7.5, 20mM $MgCl_2$), and then incubated with 5µl of gamma $^{32}P$ labeled ATP in the presence of kinase buffer for 30 minutes at room temperature. Following two washes with PBS, and one wash with thrombin buffer (50mM Tris pH8.0, 150mM NaCl, 2.5mM $CaCl_2$ and 0.04mM $MgCl_2$), GST was cleaved off the labeled ISG15 protein by addition of biotinylated thrombin

(Novagen) in presence of thrombin buffer. The supernatant was incubated with streptavidin agarose beads (Novagen) to bind the biotinylated thrombin, and passed through cleavage capture columns to get rid of the agarose beads. The $^{32}P$ labeled ISG15 was frozen or used in *in vitro* assays to detect thiol ester bond formations with UBE1L or UBCH8.

Purified GST UBE1L was obtained from Dr. Brenda Schulmann (St. Jude's Children's Hospital, Memphis, Tenneesse). GST was cleaved off UBE1l by Tev protease and dialysed (dialysis exchange buffer contained 50mM Tris pH8.0, 150mM NaCl and 0.5mM DTT) before storage at -80ºC in aliquots. GST UBCH8 was purified from bacteria, GST was cleaved using biotinylated thrombin (as described above), and UBCH8 was dialysed (see dialysis buffer above) before storage at -80ºC. Thio-ester assays were set up in 40µl reactions in T25N50 buffer containing 0.1mM DTT, 10mM $MgCl_2$ , 7.5mM ATP, 2µl of $^{32}P$ labeled ISG15, 1.4µg of purified UBE1L, 1.65µg of purified UBCH8 and 10µg of GST NS1B proteins as indicated. The reactions were incubated at room temperature for 10 minutes, after which they were boiled in SDS buffer lacking DTT to detect thiol ester bond. Buffer containing DTT was used as control to confirm that these bonds were sensitive to DTT.

## 2.3 RESULTS

### 2.3.1 Identification of ISG15 binding site on NS1B

From previous studies, it was shown that a C-terminal truncation of NS1B containing amino acids 1-104 was sufficient to bind ISG15 in *in vitro* GST pull down

assays. A smaller truncation of 1-93 amino acids, which comprised only the RNA binding domain was insufficient for this binding as were the amino acids from 95-104 (Yuan and Krug 2001). This led to the conclusion that there were regions within the 1-93 amino acids as well as between 93 and 104 amino acids that co-operatively form the surface for ISG15 interaction.

The N terminal 1-93 amino acids of NS1B forms the dsRNA binding domain whose structure shows a dimer of NS1B in a six alpha helical fold with the dsRNA groove provided by conserved basic residues in alpha helix 1 and 2 (figure 1.6) (Yin, Khan et al. 2007). We took advantage of the fact that NS1A despite having a highly similar structure of its RNA binding domain did not bind ISG15. Chimeric proteins were made in which the helices in the RNA binding domain of NS1B were swapped with those of NS1A. The mutants were tested in a GST pull down assay to test their binding to ISG15. This experiment indicated a role for helix 3 of NS1B in ISG15 binding (Zhao and Krug, data not shown). Based on the structure of the RNA binding domain, various surface exposed residues were mutated. Two kinds of mutations were made, ones in which the amino acids in NS1B were mutated to the corresponding amino acid in NS1A (figure 2.1A, lanes 5 and 6), and others in which the residues in NS1B were mutated to alanines (figure 2.1A, lanes 7-9). The binding of these mutants to ISG15 were tested in GST pull down assays (figure 2.1A). Several of these mutants involving amino acids valine 87, phenylalanine 90 and methionine 91 were defective in ISG15 binding (figure 2.1A, lanes 5-9). In addition to these residues in the RNA binding domain, phenlyalanine 100, which is between the RNA binding domain and amino acid 104 also contributed to binding (figure 2.1A, compare lanes 7 and 8). The NS1B triple alanine mutant of

V87AM91AF100A (figure 2.1A, lane 9) was chosen for future analysis. This mutation will henceforth be referred to as the 'AAA mutant'. Figure 2.1B shows a schematic representation of the N terminal 1-104 amino acids of NS1B with the residues 87, 91 and 100 indicated by a star. To confirm that the AAA mutant indeed does not bind ISG15, a reverse *in vitro* pull down experiment was conducted (figure 2.1C). Bacterially expressed GST NS1B wild type (figure 2.1C, lane 1) but not the AAA mutant protein (figure 2.1C, lane 2) was able to bind both free and conjugated ISG15 from IFN treated Hela cell extracts.

### 2.3.2 Generation of a recombinant Influenza B virus encoding an AAA mutant NS1 protein

The 'AAA' mutation identified above was then introduced into influenza B virus by the eight plasmid based reverse genetics system described previously (Hoffmann, Mahmood et al. 2002). Sequencing of the viral RNA of the recovered virus confirmed that the NS gene encoded the desired AAA mutation in NS1B. The virus was amplified by injecting purified plaques into the allantoic cavity of ten day old embryonated chicken eggs. This mutant virus shall henceforth be referred to as the AAA mutant virus.

#### *2.3.2.1 NS1 from AAA mutant virus does not bind ISG15 in infected cells*

To confirm that the NS1B protein of the AAA mutant virus indeed did not bind ISG15 in *infected* cells, we tested the binding of the virally encoded AAA mutant NS1B protein to GST-ISG15 that was transfected into 293T cells. While the NS1B from the

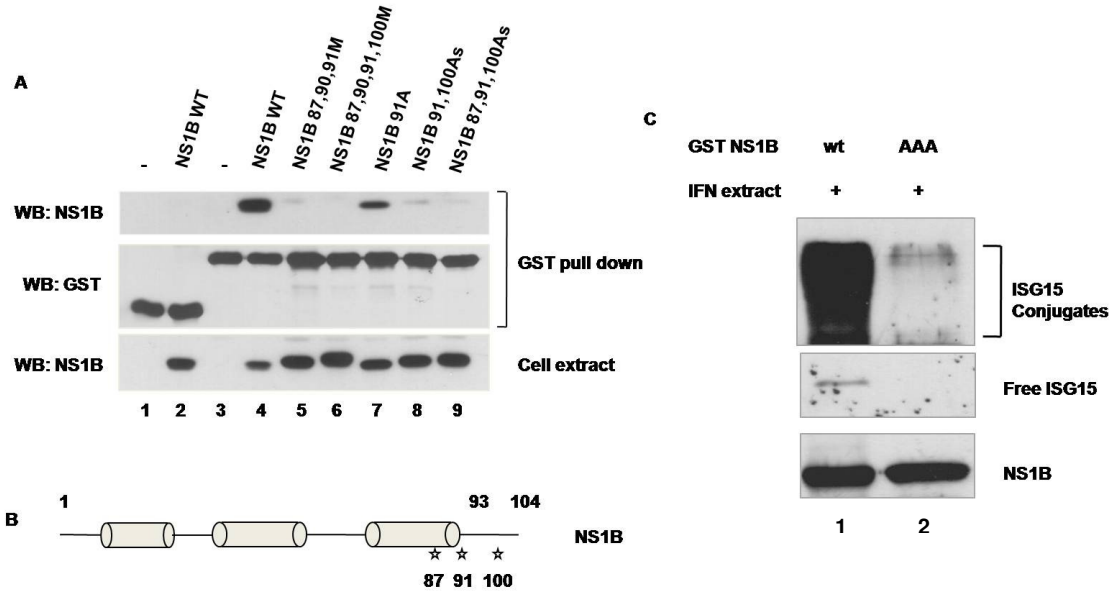



**Figure 2.1 Identification of ISG15 binding site on NS1B (AAA mutant).** A.293T cells were transfected with plasmids encoding GST (lanes 1-2) or GST-ISG15 (lanes 3-9) along with NS1B wild type (lanes 2,4) or indicated mutants (lanes 5-9). 24-48h post transfection, cell lysates were subjected to GST purification and proteins bound to beads were detected western blotting with anti NS1B or anti GST antibodies. B. Schematic representation of NS1B showing N terminal 104 amino acids. Amino acids involved in ISG15 binding are indicated by a star. C. 5 µg of bacterially purified GST NS1B wt (lane 1) or AAA mutant (lane 2) protein were bound to glutathione sepharose beads and incubated with equal amounts of extracts of Hela cells treated with 1000u of IFNβ for 40h for 2h at 4ºC. Beads were washed and boiled. Proteins bound to beads were separated by SDS PAGE an detected by western blotting with anti NS1B or anti ISG15 antibody to detect free and conjugated ISG15.

wild type virus bound the transfected GST-ISG15 (figure 2.2, lane2), NS1B from the AAA mutant virus did not (figure 2.2, lane 3), thus confirming that the NS1B from the mutant virus was unable to bind ISG15 in infected cells.

### 2.3.3 Intracellular localization of AAA mutant NS1B

To confirm that the AAA mutation does not disrupt the three dimensional protein structure of NS1B, we tested to see if AAA NS1B mutant protein still retained the correct cellular localization. NS1B accumulates in intra-nuclear compartments called the splicing speckles characterized by the staining of splicing factor SC35 (figure 2.3A). In transfection experiments in Hela Cells, we observed that the AAA mutant NS1B protein also localized to these speckles (figure 2.3B), demonstrating that this function of the NS1B protein is not compromised by the AAA mutation.

We next looked at the localization of NS1B during infection. To perform this experiment, we utilized a recombinant virus in which the NS gene encoded a NS1B protein with an N terminal 3X flag tag. This virus was developed in our laboratory and it has been shown that the N terminal tagging is not toxic to the virus (Park and Krug, unpublished results). Using the same PCR strategy (see materials and methods), the N terminal 3Xflag tagged AAA mutant NS was generated and introduced it into the virus by reverse genetics. The power of creating such a virus is the ability to use the anti flag antibody to detect the NS1B protein during infection, since the anti NS1B serum in our laboratory shows high background in immunofluroscence experiments. The N terminal 3Xflag tag also tags the NS2 protein, since NS2 message is a spliced version of the NS1B

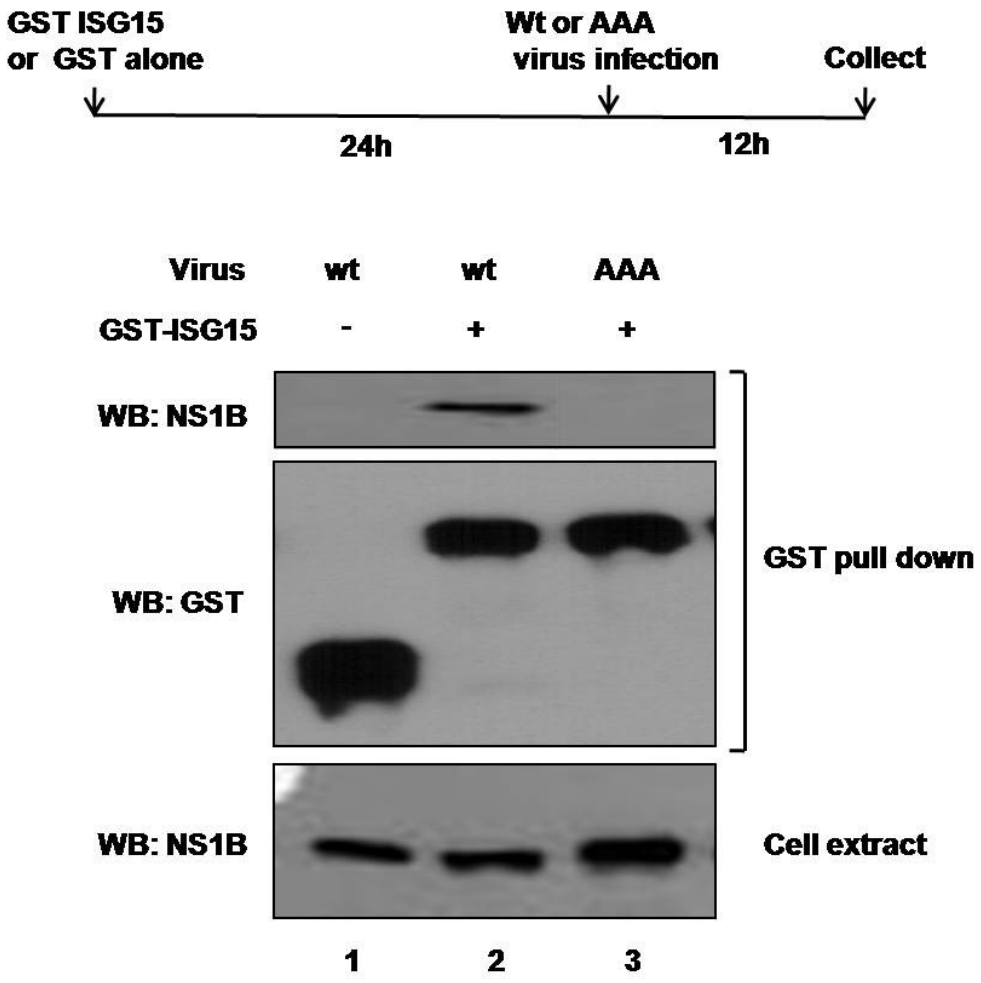



**Figure 2.2 NS1 from AAA mutant virus does not bind ISG15 in infected cells.** 293T cells were transfected with plasmid encoding GST (lane 1) or GST-ISG15 (lanes 2-3). 24h post transfection, the cells were infected with wild type (lanes 1-2) or AAA mutant (lane 3) virus. Cell lysates were prepared 12h post infection and subjected to GST purification over glutathione sepharose beads. Proteins bound to beads were separated on SDS PAGE and detected by western blot with anti NS1B or anti GST antibody.

message and the two proteins share the same N terminal eleven amino acids (Briedis and Lamb 1982). However, the NS1 protein is expressed approximately ten times more abundantly than NS2 (data not shown); hence majority of the signal coming from the anti flag antibody in immunofluroscence represents the NS1 protein. The wild type NS1B protein localizes to the nucleus early during infection (figure 2.4; 3 and 5 hours), however at later times (figure 2.4; 7 hours post infection) is present in the cytoplasm. This is unlike the NS1A protein that is present in the nucleus for most the life cycle. One of the reasons for the cytoplasmic localization of NS1B could be because it binds ISG15 which is a cytoplasmic protein. To determine if this is the case, localization of the NS1B protein of the AAA mutant virus was observed over a time course of infection. The NS1B of this virus too followed a similar localization during infection, i.e. localizing to nuclear speckles in initial times post infection and present in the cytoplasm at later stages (figure 2.4). These results indicate that the cytoplasmic localization of NS1B is not due to ISG15 binding; rather, it could be due to other as yet unidentified cytoplasmic functions of NS1B.

### 2.3.4 Re-localization of ISG15 into nucleus in influenza B infected cells

Since it was established that the localization of AAA mutant was not changed due to lack of binding ISG15, the reverse effect was tested i.e, whether NS1B has any effect on the cellular localization of ISG15. Hela cells transfected with 3xflag ISG15 showed a cytoplasmic localization for ISG15 (figure 2.5A). This was observed previously, when it was found that ISG15 localizes to intermediate filaments of the cytoskeleton

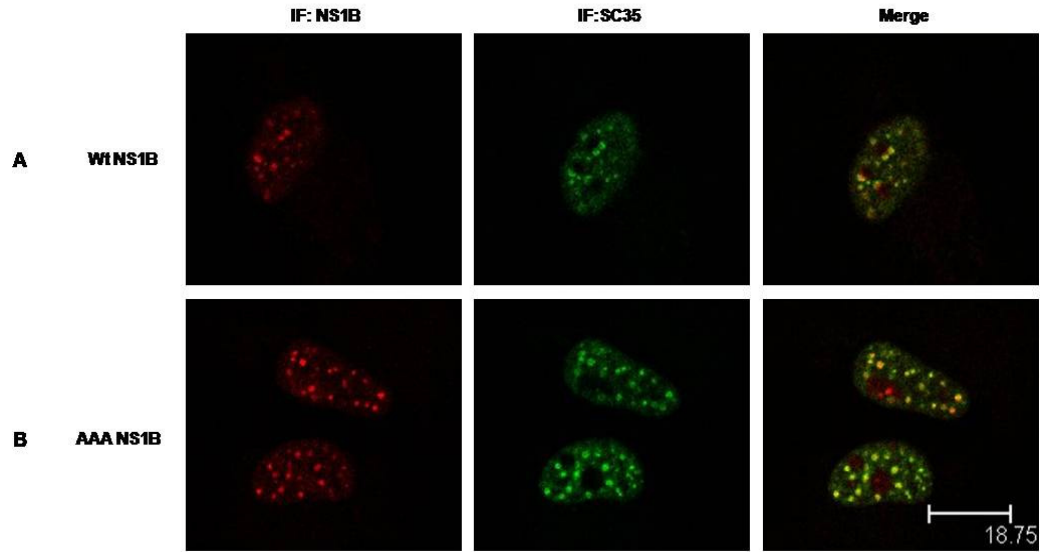



**Figure 2.3 Nuclear localization of AAA mutant NS1B in transfection.** Hela cells were transfected with plasmids encoding NS1B wild type (row A) or AAA mutant (row B). 24h post transfection, cells were fixed and subjected to immunofluroscence with rabbit anti NS1B and mouse anti SC35 antibodies. Secondary antibodies used were anti rabbit conjugated to TRITC and anti mouse conjugated to FITC. The merge column shows the merged signals from TRITC and FITC.

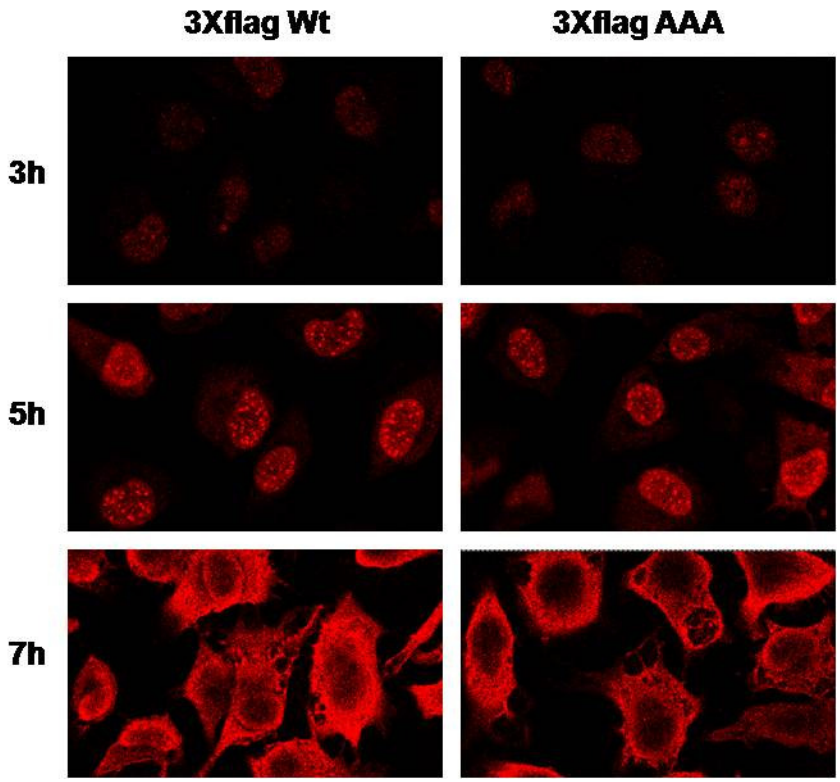



**Figure 2.4 Localization of AAA mutant NS1B during infection.** Hela cells were infected with N terminal 3X flag tagged wild type or AAA mutant virus at an moi of 2-5. At 3, 5 and 7 hours post infection, the cells were fixed and immunofluroscence was performed with mouse anti flag antibody to detect NS1B protein and anti mouse TRITC as secondary antibody.

(Loeb and Haas 1994). The transfected cells were then infected with wild type (figure 2.5B) or AAA mutant virus (figure 2.5C). As can be seen from the wild type infected cells, the ISG15 was dramatically re-localized into the nucleus, with very little cytoplasmic staining visible (figure 2.5B). Within the nucleus, ISG15 could clearly be seen enriched in SC35 speckles. We confirm that this re-localization of ISG15 is due to binding NS1B by showing that ISG15 is predominantly cytoplasmic during infection of the AAA mutant virus (figure 2.5C). To detect infected cells, the anti-Memphis antibody (kindly provided by Dr. McCullers, St. Jude's children's hospital, Memphis) which was raised against B/Memphis virus and detects all the structural proteins of influenza B virus, was used.

In our experiments, we have observed that NS1B not only binds free ISG15, but also binds pre-formed ISG15 conjugates when the latter are present (figure 2.1C). Therefore, it is reasonable to predict that the wild type NS1B will be able to also re-localize the conjugated ISG15 into the intra-nuclear SC35 speckles.

### 2.3.5 AAA mutant virus is not attenuated in multiple cycle growth in MDCK cells

The multiple cycle growth characteristics of the AAA mutant virus in MDCK cells were studied. MDCK cells are derived from canine kidney cells and are the predominant cells used in the field to grow influenza viruses in tissue culture. To study the multiple cycle growth of AAA virus, MDCK cells were infected with either wild type or AAA mutant influenza B virus at a low multiplicity of infection (moi=0.001) (figure

2.6). The AAA mutant virus is neither attenuated in growth kinetics nor final virus yield as compared to the wild type virus (figure 2.6).

### 2.3.6 Screen for a suitable human tissue culture system to study influenza viruses

The AAA mutant virus is not attenuated in MDCK cells which are canine kidney cells (figure 2.6). One of the reasons for the lack of attenuation could be that MDCK cells are not a suitable system to study the effect of ISG15 influenza B viruses. Influenza B virus is almost an exclusively human virus, unlike influenza A viruses which cause disease in a wide variety of mammals and birds. Further, the enzymes required for conjugation of ISG15, i.e UBE1L, UBCH8 and HERC5 as well as the targets have been characterized in human cells. Also the E3 enzyme, HERC5, is absent in certain other mammals like mice. Finally, but perhaps most importantly, NS1B does not bind canine ISG15 (refer section 2.4). All these reasons led us to search for a good human cell culture system to study the AAA mutant virus.

Very few cell types support multiple cycle influenza virus growth in tissue culture. This restriction arises due to the requirement for the cleavage of the viral membrane protein, hemagglutinin (HA), in the absence of which the released viral particles are unable to re-infect the cells (for a detailed discussion, refer section 2.5.2). MDCK cells are the predominant cells used to study influenza viruses in tissue culture, because of their ability to withstand the concentrations of trypsin needed for achieving multiple cycle growth. In our screen for an ideal human cell line, we looked for

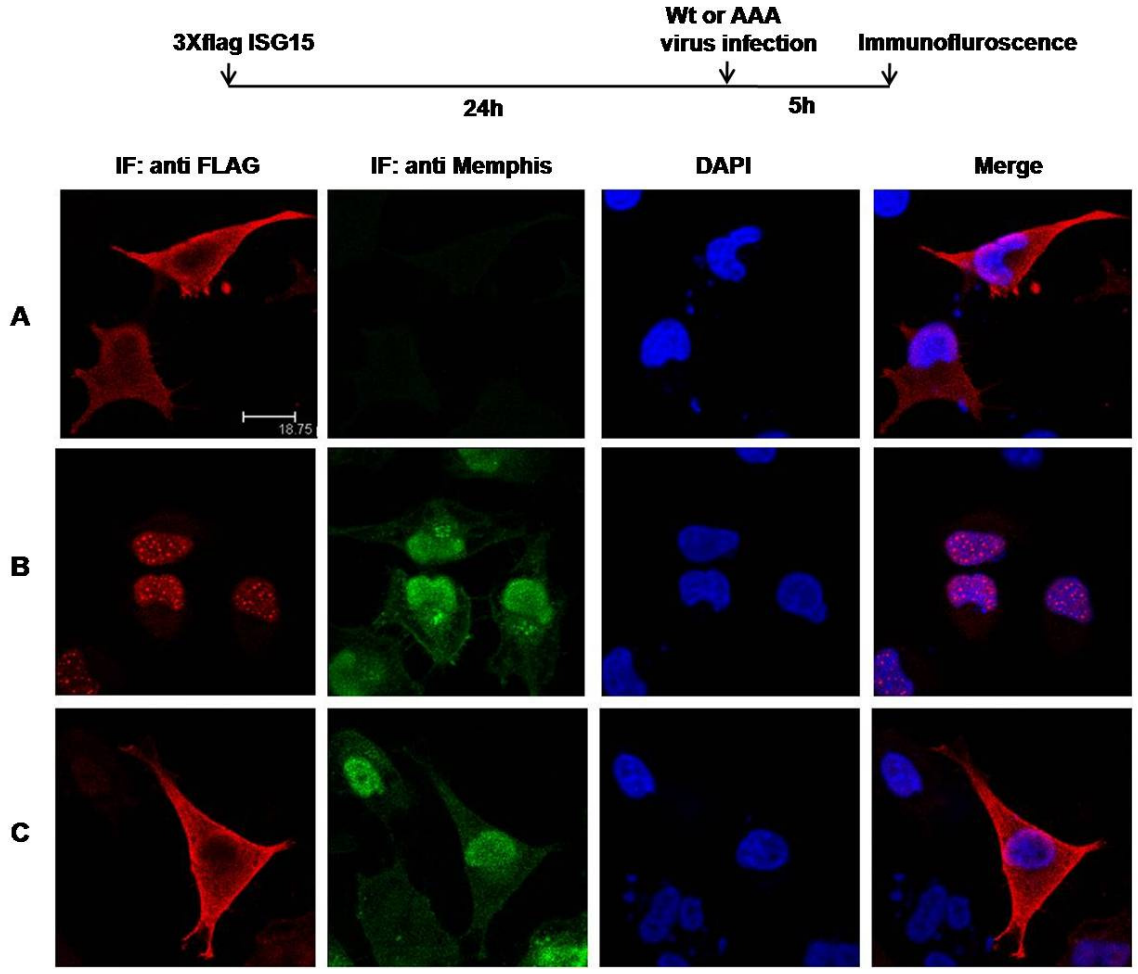



**Figure 2.5 Localization of ISG15 during influenza B virus infection.** Hela cells were transfected with plasmid encoding 3XFlag ISG15. 24h post transfection, cells were mock infected (panel A), or infected with wild type (panel B) or AAA mutant (panel C) influenza B virus. 5h post infection, cells were fixed and subjected to immunofluroscence with mouse anti flag and ferret anti Memphis antibody to detect flag tagged ISG15 and influenza viral proteins respectively. Anti mouse TRITC and anti ferret FITC were used as secondary antibodies. DAPI staining was performed to identify the nuclei. The merge panel shows the merged signals from TRITC and DAPI signals.

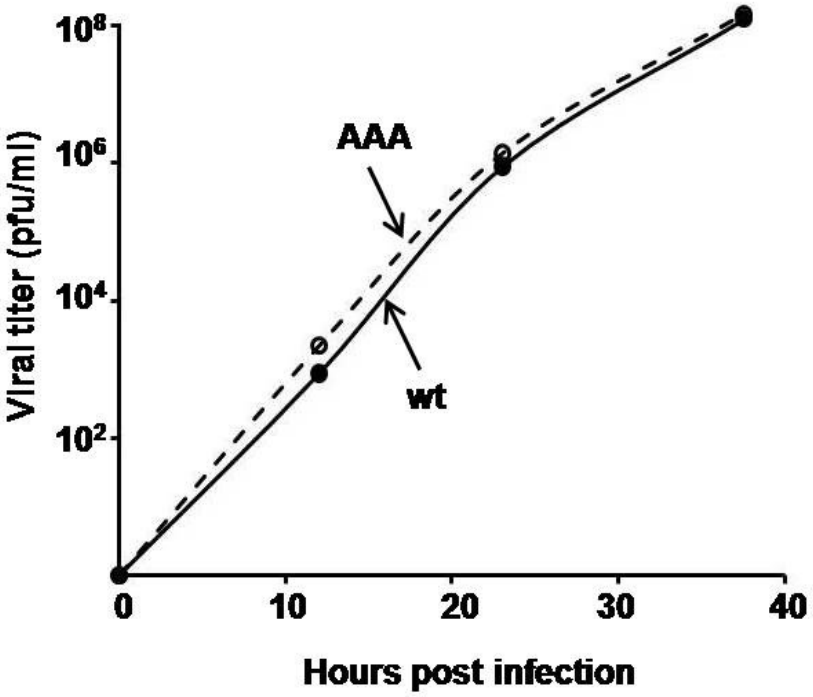



**Figure 2.6 Multiple cycle growth of AAA mutant virus in MDCK cells.** 80-90% confluent MDCK cells in 60mm dishes were infected with wild type or AAA mutant virus at a multiplicity of infection (moi) of 0.001. 1h post adsorption, the cells were washed and over layed with opti-MEM containing 2.5μg/ml N acetyl trypsin and 1% PSG. At specific points after infection, the cell supernatants were collected and assayed for viral titer by plaque assay.

responsiveness of the cell to IFN in terms of ability to induce ISG15 and its conjugation as well as the ability of the cell line to support multiple cycle growth of influenza virus.

An array of human cell lines was tested for their responsiveness to IFN in terms of induction of ISG15 and its conjugation. 293T, A549, Hela tet-on and Calu-3 cells were treated with 100 or 1000units of IFN for 36h at 37ºC (figure 2.7). IFN induced free ISG15 in 293T cells, but no conjugation was detected. It could be that the enzyme(s) responsible for ISG15 conjugation are defective or absent in these cells. In contrast, IFN induced robust ISG15 and its conjugation in A549 and Hela tet-on cells. In Calu-3 cells, IFN induced less robust ISG15 conjugation than in A549 or Hela tet-on cells. Also, ISG15 conjugation can be detected in A549 and Hela tet-on cells but not Calu-3 with a shorter 24h treatment with IFN (data not shown).

Next, the growth characteristics of wild type influenza B virus in A549, Hela tet-on and Calu-3 cells were studied (figure 2.8). MDCK cells were included as a positive control. Viral growth reached titers of $10^8$ pfu/ml in MDCK. Since A549 and Hela tet-on cells are very sensitive to trypsin, they were grown on collagen coated plates to enable them to bind tighter, and N acetyl trypsin (NAT) was added at a reduced concentration of 0.1 μg/ml instead of 2.5 μg/ml that is added to MDCK cells. Infection of these two cell lines with wild type influenza B virus at a moi of 0.001 failed to produce any virus (data not shown). When they were infected with influenza B virus at a moi of 0.01, virus grew to titers of ~$10^5$pfu/ml. Only Calu-3 cells were able to support growth of virus to titers of ~$10^8$pfu/ml, when infected with a moi of 0.001. The growth of virus in Calu-3 cells more closely compared to the viral growth in the control MDCK cells. Calu-3 cells also

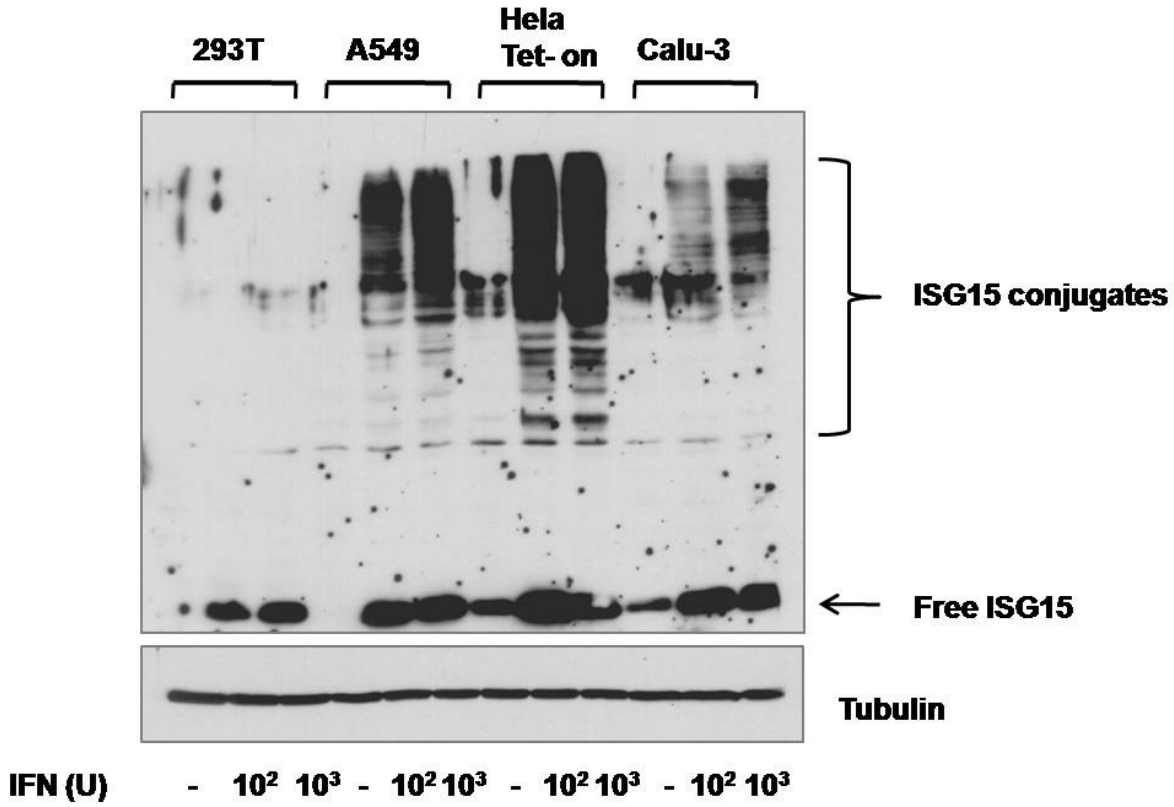



**Figure 2.7 Induction of ISG15 conjugation in human cell lines.** 293T, A549, Hela tet-on and Calu-3 cells were treated with 100 or 1000 units of human IFNβ for 36h at 37ºC. Cell lysates were subjected to western blot with anti- ISG15 antibody to detect the induction of free and conjugated ISG15. Western blot with anti- tubulin antibody serves as loading control.

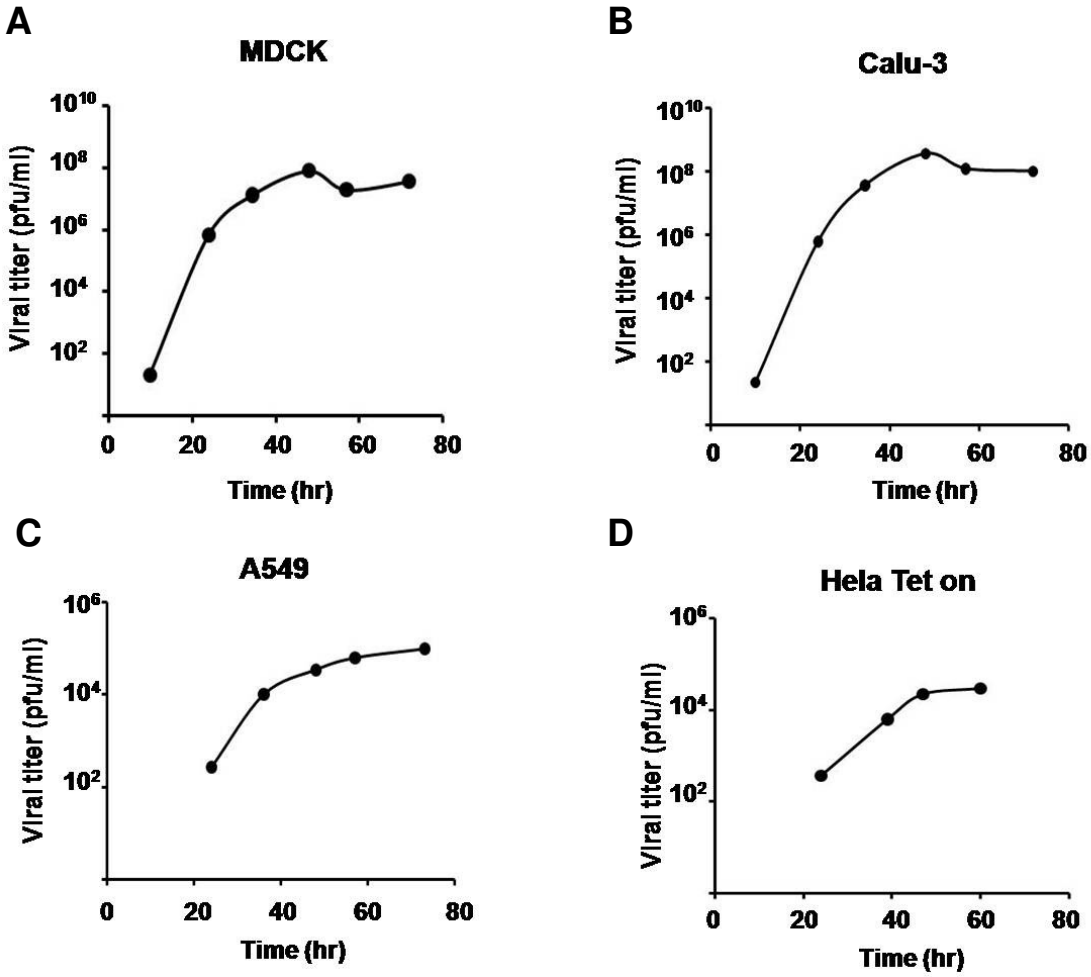



**Figure 2.8 Multiple cycle growth of influenza B virus in human cell lines.** 80-90% confluent MDCK and Calu-3 cells were infected with wild type B/Yamanashi/98 virus at a moi of 0.001. A549 and Hela tet-on cells were grown on collagen coated plates and infected at a moi of 0.01. After 1h of adsorption, cells were washed and over layed with opti-MEM supplemented with 2.5µg/ml (MDCK and Calu-3) or 0.1µg/ml (A549 and Hela tet-on) N acetyl trypsin together with 1% PSG. For A549 and Hela tet-on, NAT was supplemented at 36h post infection. At different times post infection, cell supernatant was collected and assayed for viral titer by plaque assay.

supported efficient virus growth in the absence of exogenously added NAT (data not shown). It is possible that these cells express a trypsin- like protease that is able to cleave the viral HA protein. Therefore, for future characterization of the AAA mutant virus, Calu-3 cells were chosen since they alone among all the cell types tested supported the appropriate multiple cycle growth of influenza B virus.

### 2.3.7 Characterization of AAA mutant virus in calu-3 cells

Calu-3 cells were infected at a moi of 0.001 with either wild type or AAA mutant virus (figure 2.9), and multiple cycle growth monitored for each virus. The AAA mutant virus grows at approximately ten fold slower rate than the wild type virus, as shown by the 12 hour ($1.68*10^2$ pfu/ml for AAA virus and $1.3*10^3$ pfu/ml for wild type virus) and 24 hour ($2.9*10^4$ pfu/ml for AAA virus and $1.9*10^5$ pfu/ml for wild type virus) time points. At 38 hours post infection, the rate of the wild type virus is 5 times higher than that of the AAA mutant virus. These results are in contrast to the results in MDCK cells where the AAA mutant virus and wild type virus grew at same rates (figure 2.6).

### 2.3.8 ISG15 shows species specific variation

One of the intriguing features of ISG15 is its great variation in sequence across species. A sequence comparison between human, rhesus monkey, canine and mouse ISG15 (figure 2.10) shows that canine and mouse ISG15 are respectively only 69% and 65% identical to human ISG15. However, rhesus monkey ISG15 is much more similar to human ISG15, showing 92% identity to the human counterpart (calculation of %

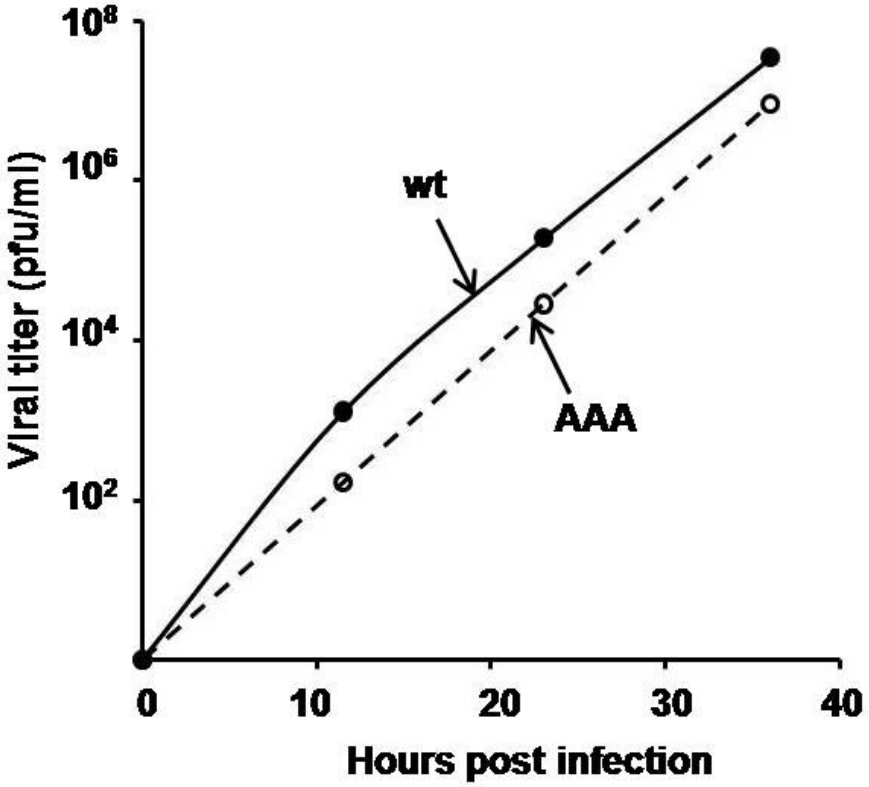



**Figure 2.9 Multiple cycle growth of AAA mutant virus in Calu-3 cells.** 75-90% confluent calu-3 cells in 60mm dishes were infected with wild type or AAA mutant virus at a moi of 0.001. After 1h of adsorption, cells were washed and over-layed with opti-MEM supplemented with 2.5 μg/ml NAT. Cell supernatant was collected at various time points and assayed for viral titer by plaque assay.

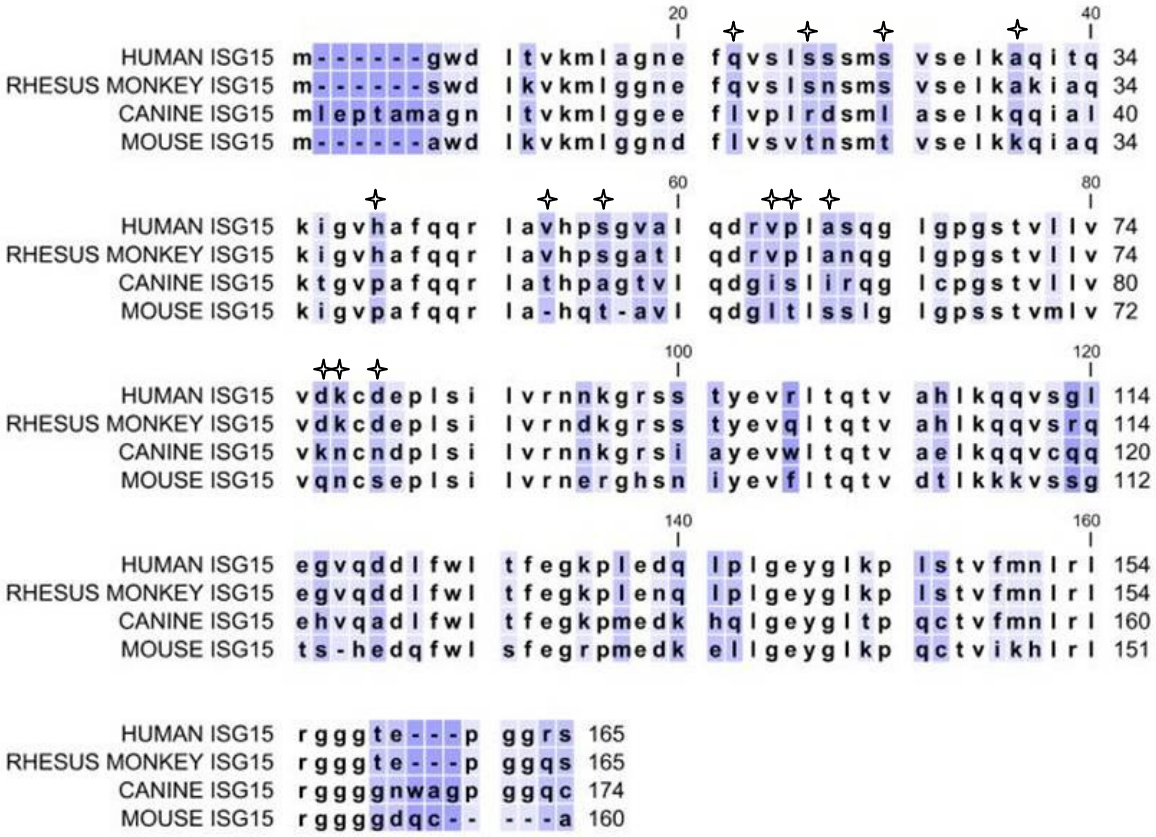



**Figure 2.10 A sequence alignment of ISG15 orthologs.** ISG15 protein sequences from human, rhesus monkey, dog and mouse were analyzed by multiple alignment using CLC workbench 4.0. Variant residues between the different species are shaded in blue with the most intensely shaded residues differing the most between the four species. Un-highlighted residues are conserved between the species. Stars denote residues in the N terminal domain that are conserved between human and Rhesus monkey but differ in canine or mouse ISG15.

identitity took into consideration sequences until the LRLRGG motif for all ISG15 homologs). The sequence variation of ISG15 is unlike ubiquitin, which is remarkably conserved between different species and shows great conservation from yeast to humans. Therefore, it is reasonable to predict that there may exist differences in the functions of ISG15 from different species.

### 2.3.9 NS1B binds ISG15 in a species specific manner

The sequence variation in ISG15, coupled with the fact that AAA mutant virus was not attenuated in canine MDCK cells led us to test if ISG15 from different mammalian species all bind NS1B. Mouse ISG15 was obtained from Dr. Jon Huibregtse's laboratory. Canine ISG15 (Hsiang, T.Y) and monkey ISG15 were cloned from MDCK and Cos7 cells respectively after inducing ISG15 expression with poly I:C. Sequence analysis confirmed that these were indeed the canine and monkey ISG15 proteins. In an *in vivo* GST binding asay, the binding of NS1B to N terminal GST tagged human, mouse, canine or monkey ISG15 was tested (figure 2.11). As expected, NS1B bound human ISG15 very well (figure 2.11, lane 2). However, neither the mouse nor canine ISG15 bound NS1B (figure 2.11, lanes 3 and 4). Monkey ISG15, which is much closer to human ISG15 in terms of sequence, bound NS1B as well as its human counterpart (figure 2.11, lane 5). These results show that the interaction between ISG15 and NS1B is species specific, and may explain the lack of attenuation of the AAA mutant virus in MDCK cells. Because canine ISG15 does not bind NS1B, knocking out the binding between these two proteins in canine cells has no consequence for the virus. Our

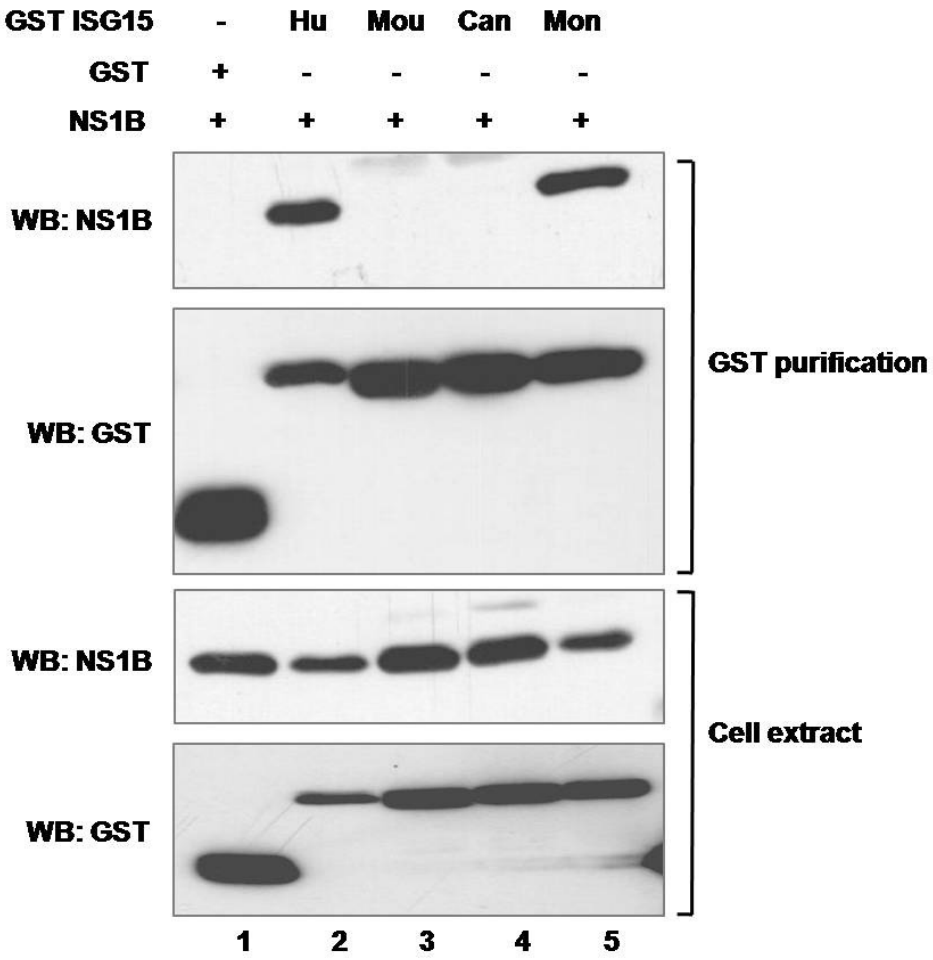



**Figure 2.11 Species specific interaction of ISG15 with NS1B.** 293T cells were transfected with plasmids encoding GST alone (lane 1) or GST fused to human (lane 2), mouse (lane 3), canine (lane 5) or monkey (lane 5) ISG15, along with NS1B. 24-36h post transfection, cell lysates were purified over glutathione sepharose beads. After washing, the beads were boiled and subjected to western blotting with anti NS1B or anti GST anti sera to detect NS1B and GST fusion proteins respectively.

results also explain the rescue of wild type influenza B virus in ISG15$^{-/-}$ mice. Because NS1B does not bind mouse ISG15, it cannot inhibit the latter's activity.

### 2.3.10 Monkey ISG15 as an alternate model system to study influenza B viruses

The fact that monkey ISG15 bound NS1B suggested the possibility of using monkey systems as a suitable model for studying influenza B virus. For this, it would be important to establish that monkey ISG15 is highly similar in structure to human ISG15. Since a crystal structure of monkey ISG15 is not available, we undertook to see if the interaction between NS1B and monkey ISG15 is similar to the interaction between NS1B and human ISG15.

ISG15 has two 'ubiquitin- like' domains (Narasimhan, Wang et al. 2005). The C terminal lobe recognizes UBE1L and contains the C terminal LRLRGG motif that is conjugated to target proteins (Chang, Yan et al. 2008). To determine if NS1B binds ISG15 on its N or C terminal domains, fusion proteins of GST tagged to the N or C termini were constructed and tested for their ability to bind NS1B in an *in vivo* GST pull down assay (figure 2.12). The N terminal lobe of ISG15 is sufficient to bind NS1B as well as the full length protein while the C terminus does not bind (figure 2.12, compare lanes 2, 3 and 4). This observation was also reported by another group of researchers (Chang, Yan et al. 2008). To test if the N terminus of owm ISG15 is similarly sufficient to bind NS1B, GST fused to the N and C termini of owm ISG15 were constructed and

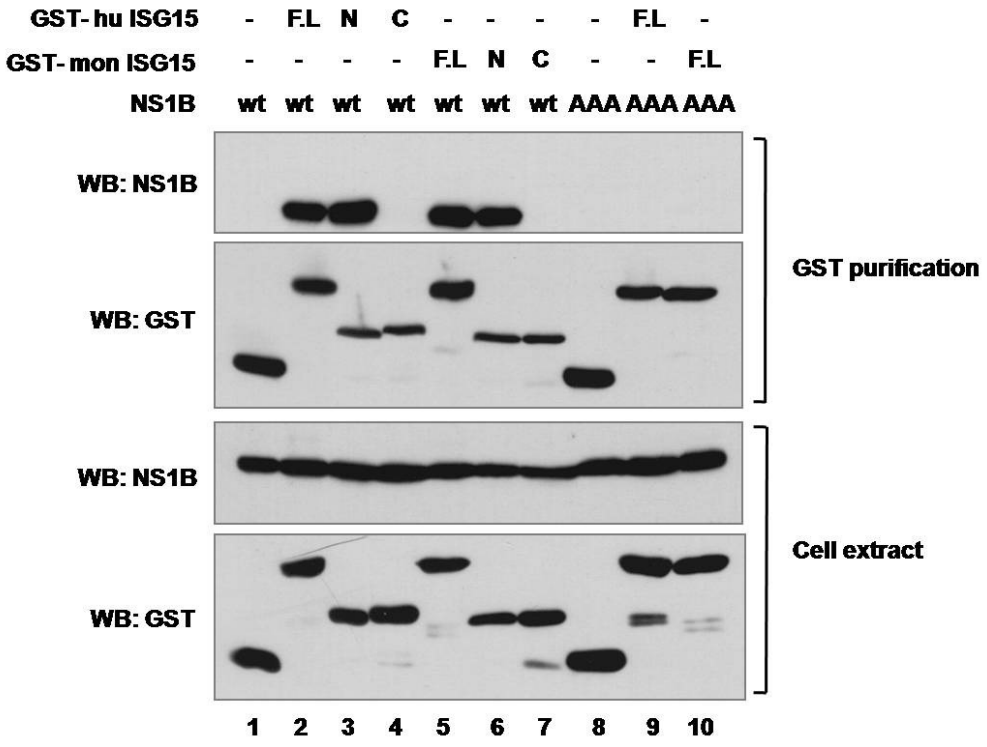



**Figure 2.12 Characterization of interaction between NS1B and monkey ISG15**. A.293T cells were transfected with plasmids encoding GST alone (lanes 1 and 8) or GST fused to human ISG15 full length (lane 2), human ISG15 N and C lobes (lanes 3-4), monkey ISG15 full length (lane 5) or monkey ISG15 N and C lobes (lanes 6-7) along with NS1B wild type (lanes 1-7) or AAA mutant (lanes 8-10). 24-36h post transfection, cells lysates were purified over glutathione sepharose beads and subjected to western blotting with anti NS1B or anti GST antisera to detect NS1B and GST fusion proteins.

tested for their ability to bind NS1B. The N terminus of monkey ISG15 binds NS1B as well as the full length monkey ISG15 protein (figure 2.12, compare lanes 5, 6 and 7). Further, the AAA mutant NS1B that does not bind human ISG15 also lacks binding capability to monkey ISG15 (figure 2.12, lanes 9 and 10). These results taken together suggest that the interaction surface between NS1B and monkey ISG15 is similar to its interaction with human ISG15.

### 2.3.11 Role of the hinge region of ISG15 in NS1B recognition

In order to map the binding site of NS1B on ISG15, we took advantage of the fact that the N terminus of ISG15 is sufficient to bind NS1B. Our first approach was to focus on the variant residues between ISG15 orthologs. A sequence comparison of the N termini along with hinge of human, rhesus monkey, canine and mouse ISG15 showed a total of thirteen residues that were identical to human and rhesus monkey but different from mouse and canine (figure 2.10, denoted by stars). However, mutation of each of these residues by themselves or in combination in human ISG15 failed to disrupt NS1B binding (data not shown).

In our second approach, we identified the minimum human sequence that when introduced into the mouse full length ISG15 was able to impart NS1B binding to the mouse protein. A series of human-mouse ISG15 chimeric proteins were constructed (figure 2.13A) and tested for their ability to bind to NS1B (figure 2.13B). The introduction of amino acids 37 to 80 from human ISG15 into the corresponding region of the full length mouse ISG15 was sufficient to impart NS1B binding to the mouse protein (figure 2.13B, lane 3). The region from amino acids 37 to 80 comprises part of the N

terminal domain (37-74 amino acids of human ISG15) and the hinge region (amino acids 75-80). Replacement of the mouse hinge with the human hinge alone was able to impart partial NS1B binding (figure 2.13B, lane 5), while the replacement of the region of the N terminal domain (amino acids 37-74) was unable to do so (figure 2.13B, lane 4). This suggests that the hinge region is crucial in the binding of ISG15 by NS1B. However, the human hinge region by itself was insufficient to recover complete binding for the mouse full length protein (figure 2.13B compare lanes 1 and 5). A dramatic increase in binding occurred when amino acids 63 to 74 of the N terminal domain of human ISG15 was added in addition to the hinge of the mouse ISG15 (figure 2.13B lane 6). The surprising feature of this region is that the sequence is almost completely conserved between canine and human ISG15 with the exception of a glycine at position 66 of human ISG15 that is replaced by a cysteine in canine ISG15 (figure 2.14A). We therefore hypothesized that replacement of canine ISG15 with just the hinge of human ISG15 should be able to restore efficient binding capability to the canine ortholog. Indeed, this was the case, and unlike mouse ISG15, canine ISG15 with hinge region from human protein was able to restore NS1B binding almost as good as human ISG15 (figure 2.14B, compare lanes 1 and 3). Further, loss of function mutations in the human hinge also provided more evidence for the role of hinge in NS1B binding (figure 2.15). When the three differing hinge residues (amino acids 76, 77 and 79) of human ISG15 were mutated to those found in mouse hinge, this triple mutant protein no longer was able to bind NS1B (figure 2.15, lane 2). Further, single mutations of each of these amino acids to those of mouse (D76Q,

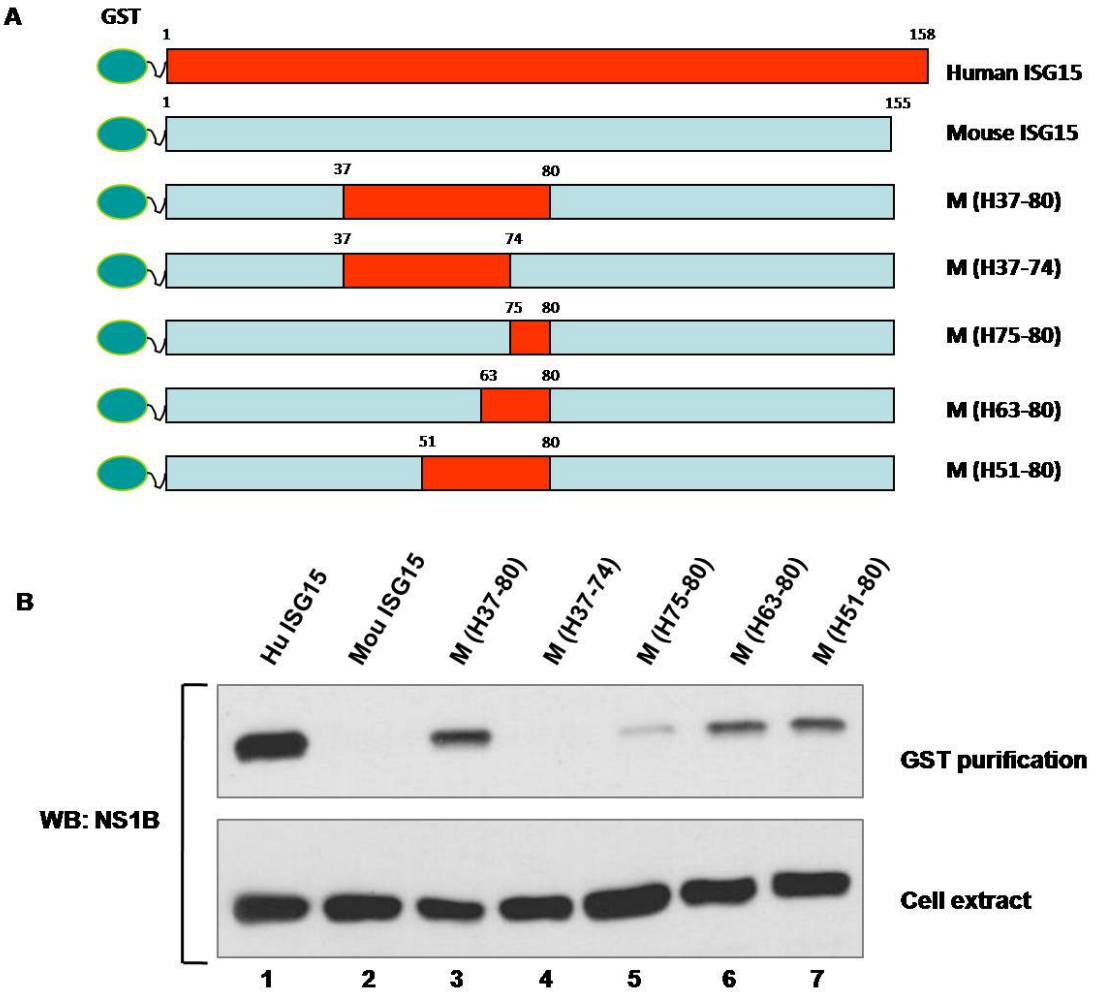



**Figure 2.13 Binding of human-mouse ISG15 chimeric proteins to NS1B.** A. Schematic representation of the human-mouse ISG15 chimeric proteins generated. Indicated are the amino acids of human ISG15 that replaced the corresponding regions of mouse ISG15. Proteins were tagged at the N terminus with GST. Not drawn to scale. B. 293T cells were transfected with plasmid encoding NS1B and GST tagged full length human (lane 1) or mouse (lane 2) or various chimeras (lanes 3-7). 24h post transfection, cell lysates were subjected to purification over glutathione sepharose beads. Beads were boiled and subjected to western blotting with anti-NS1B antibody. NS1B expression in cell extract prior to purification is shown as control.

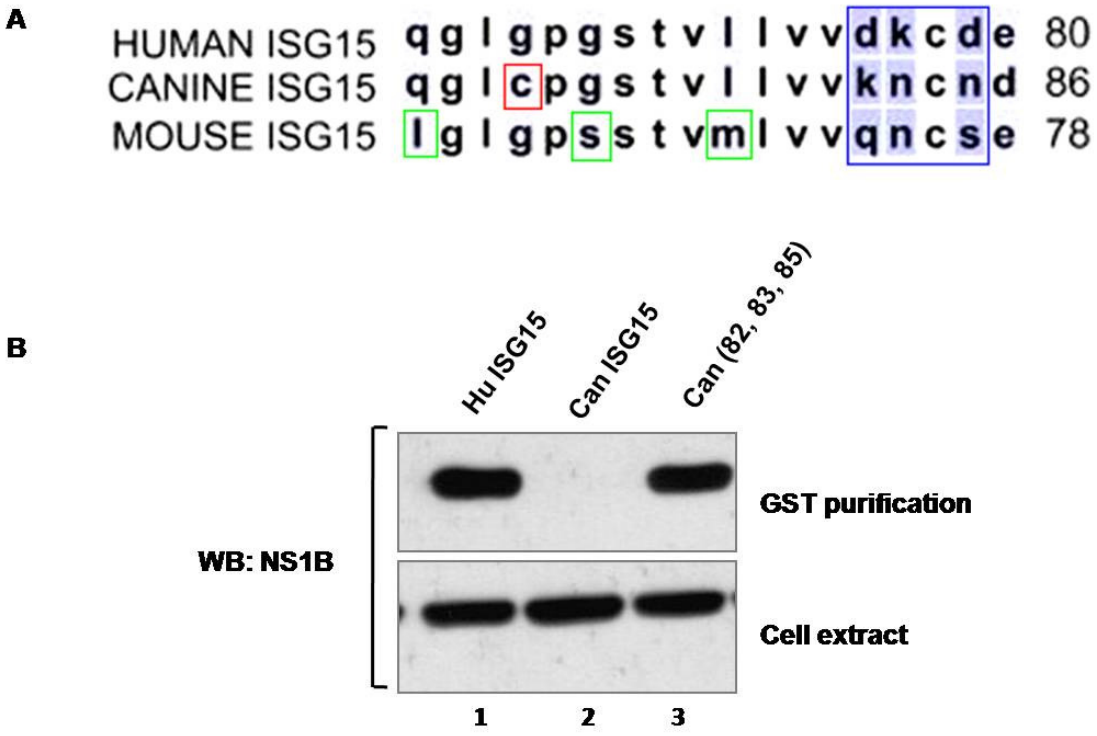



**Figure 2.14 Minimum requirement for canine ISG15 to bind NS1B.** A.Amino acids 63-80 of human ISG15 are aligned with the corresponding regions of canine and mouse ISG15. Blue box represents hinge region, red box represents the single differing residue between human and canine ISG15 and green boxes represent differing residues between human and mouse ISG15. B. 293T cells were transfected with plasmids encoding NS1B along with GST tagged human ISG15 (lane 1), canine ISG15 (lane 2) or canine ISG15 with amino acids 82, 83 and 85 changed to corresponding amino acids of human ISG15 (lane 3). Cell lysates were subjected to GST purification and western blotting with anti NS1B antibody. NS1B expression in cell extracts prior to purification serves as control.

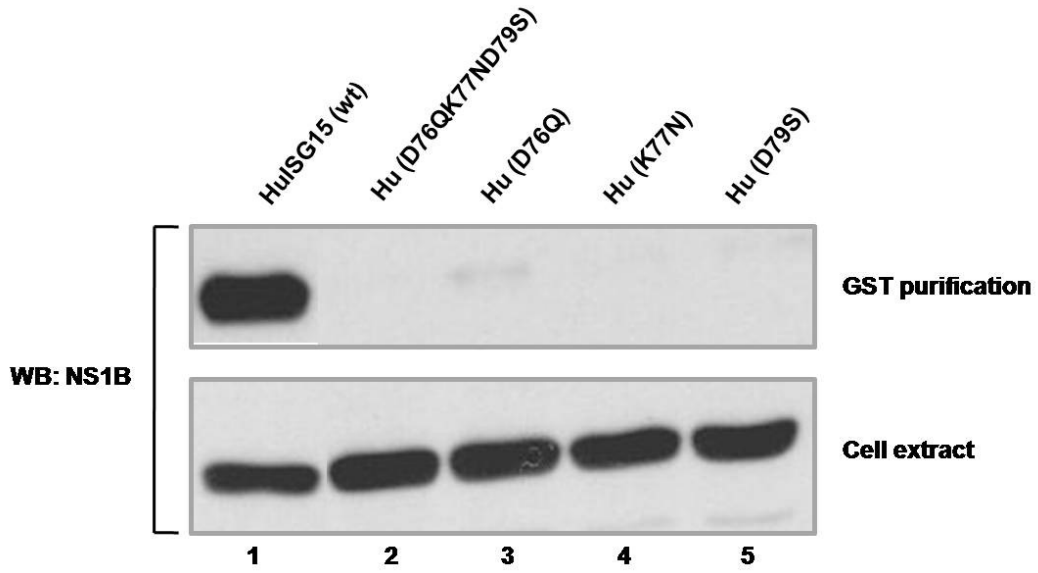



**Figure 2.15 Human ISG15 hinge mutants cannot bind NS1B.** 293T cells were transfected with plasmid encoding NS1B along with plasmid encoding GST human ISG15 wild type (lane 1), or mutants with various residues in the hinge region mutated (lanes 2-5). 24h post transfection, cell extracts were subjected to purification over glutathione sepharose beads and western blotting with anti NS1B antibody. NS1B expression in cell extracts prior to purification serves as control.

K77N or D79S) were also sufficient to disrupt NS1B binding (figure 2.15, lanes 3-5). The above results taken together strongly suggest that the hinge region of ISG15 is one of the major determining factors of the species specific nature of NS1B binding.

### 2.3.12 Effect of NS1B on ISG15 conjugation

#### *2.3.12.1 NS1B does not inhibit in vitro ISG15 thio-ester bond formation with UBE1L and UBCH8*

An early observation in our laboratory was that although free ISG15 is induced in influenza B virus infection, ISG15 conjugation does not occur. In an *in vitro* assay using extracts from baculovirus sf9 cells expressing UBE1L or IFN treated A549 cells, it was found that the N terminal 1-145 amino acids of NS1B inhibited the first step in the conjugation pathway i.e formation of the UBE1L~ISG15 thio-ester bond (Yuan and Krug 2001). Our efforts to repeat that result using purified UBE1L (kindly provided by Dr. Brenda Schulmann) proved unsuccessful. To detect UBE1L and UBCH8 thio-ester bond formations with ISG15, purified UBE1L and UBCH8 were incubated with $^{32}$P labeled ISG15. The reactions were stopped, boiled in buffer lacking DTT and analyzed by SDS PAGE. The ISG15~UBE1L thio-ester bond was identified by its dependence on the presence of UBE1L (figure 2.16A compare lanes 1 and 2) and its sensitivity to DTT (figure 2.16A, compare lanes 2 and 5). Similarly the UBCH8~ISG15 thio-ester bond was observable upon the addition of UBCH8 (figure 2.16A, lane 3) and disappeared upon addition of DTT (figure 2.16A, lane 6). However, there was no effect on either the UBE1L~ISG15 or UBCH8~ISG15 thio-ester bond formations upon the addition of

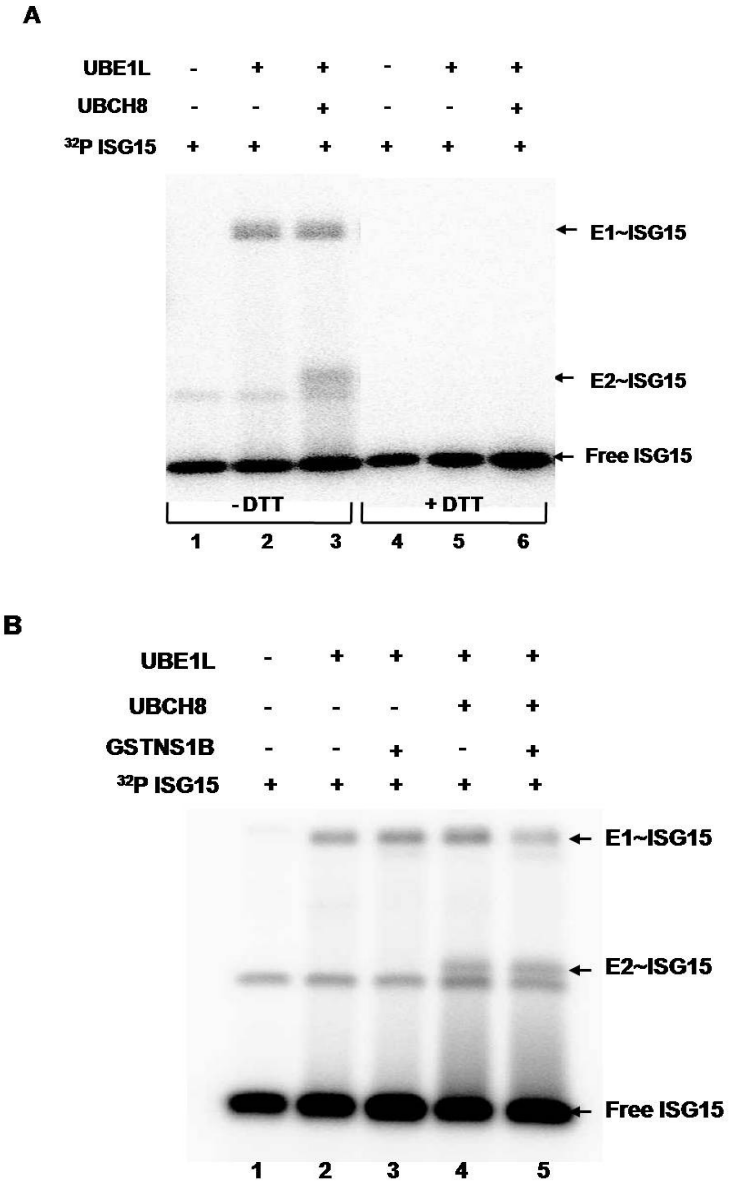



**Figure 2.16 NS1B does not inhibit ISG15~UBEIL and ISG15~UBCH8 reactions.** A. 0.05 μg of $^{32}$P labeled ISG15 was incubated with 1.4 μg purified UBE1L and 1.65 μg of purified UBCH8 in the presence of buffer containing ATP for 10 minutes. Samples were prepared in buffer lacking DTT (lanes 1-3) to detect E1 and E2 thioester bonds with ISG15. These bonds disappear upon addition of DTT (lanes 4-6). B. 10 μg of GST NS1B was pre-incubated with $^{32}$P labeled ISG15 (lanes 3 and 5) prior to incubation with UBE1L or UBCH8.

purified NS1B (figure 2.16B, compare lanes 2 and 3; 4 and 5), even when NS1B was supplied at a molar excess of 400 as compared to ISG15. These results were subsequently confirmed by another group of researchers (Chang, Yan et al. 2008).

#### *2.3.12.2 Effect of NS1B on ISG15 conjugation in vivo*

We next studied the effect of NS1B on ISG15 and its conjugation in transfection experiments. When wild type NS1B was co-expressed with 3xflag human ISG15 along with UBE1L, UBCH8 and HERC5 in a quintuple transfection experiment in 293T cells, it decreased the intracellular levels of ISG15 conjugates (figure 2.17A, compare lanes 1 and 2). To test if ISG15 binding is the only cause of this inhibition, the plasmid expressing the AAA mutant NS1B instead of the wild type NS1B was transfected (figure 2.17A, lane 3). Surprisingly, conjugation was only partially restored in the presence of the AAA mutant NS1B (conjugation was 40-50% of the conjugation that occurred in the absence of NS1B). Similarly, wild type NS1B was able to reduce the levels of owm ISG15 conjugates in Hela tet-on cells (figure 2.17B, lanes 3 and 5). Again, the levels were partially restored in the presence of the AAA mutant NS1B (figure 2.17B, lanes 4 and 6). In a reverse experiment, we tested the effect of wild type NS1B on the hinge mutant of human ISG15 which does not bind NS1B (figure 2.17C). However, its conjugation is still reduced in the presence of wild type NS1B (figure 2.17C, compare lanes 3 and 4). These results taken together suggest that there is another mechanism besides binding ISG15 by which NS1B inhibits ISG15 conjugation. Surprisingly, the hinge mutant ISG15 was conjugated to a poorer extent when compared to the wild type ISG15 (figure 2.17C, compare lanes 1 and 3).

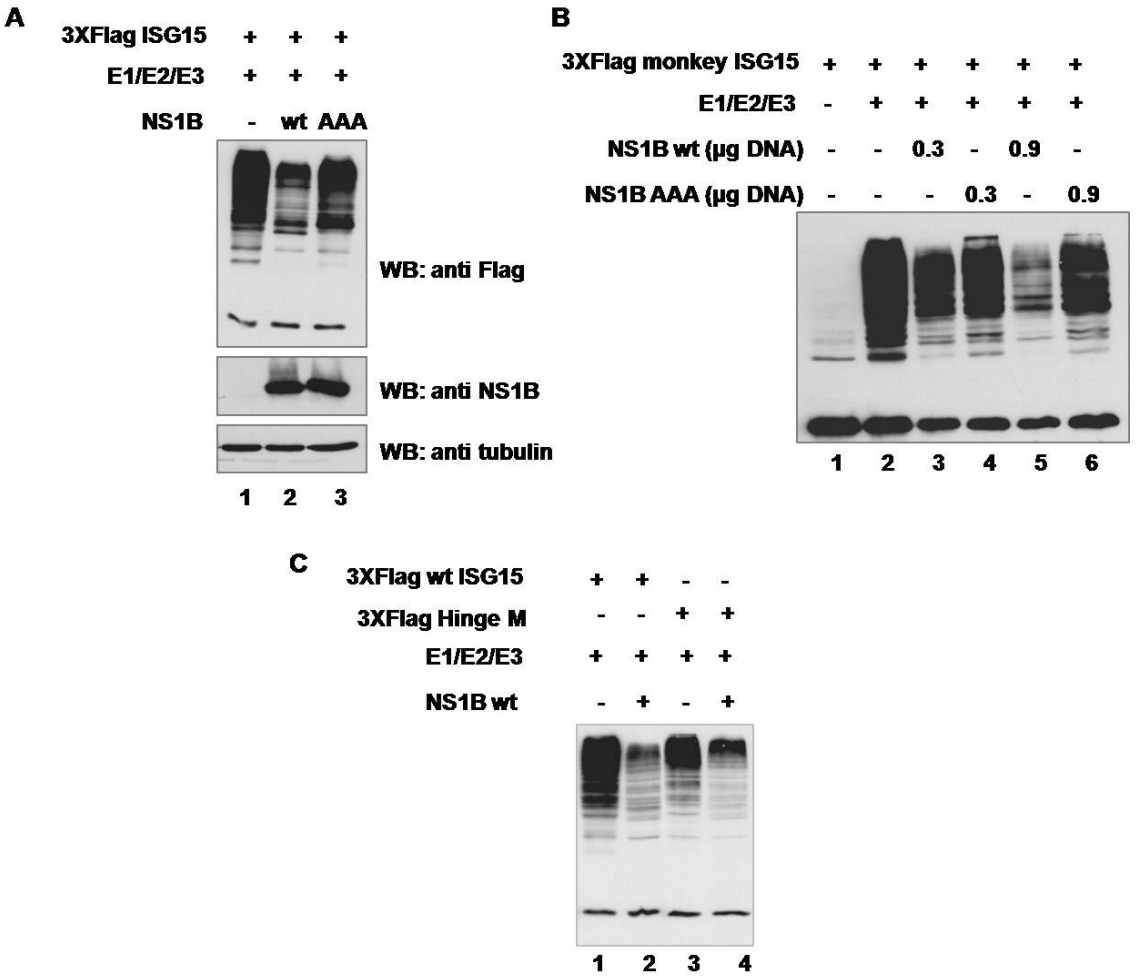



**Figure 2.17Effect of NS1B on ISG15 conjugation *in vivo*.** A.293T cells were transfected with plasmids encoding 3xflag huISG15 along with plasmids encoding the conjugation enzymes (E1, E2 and E3). Plasmids encoding wild type (lane 2) or AAA mutant (lane 3) NS1B was also co-transfected with the above. B. Hela tet-on cells were transfected with plasmid encoding 3xflag monkey ISG15 without (lane1) or with (lanes 2-6) plasmids encoding conjugation enzymes. NS1B wt (lanes 3,5) or AAA mutant (lanes 4,6) were also transfected in increasing amounts. C. 293T cells were co-transfected with plasmid encoding 3xflag hu ISG15 wt (lanes 1-2) or 3xflag huISG15 (76,77,79) hinge mutant (lanes 3-4) along with conjugation enzymes in absence (lanes 1,3) or presence (lanes 2,4) of NS1B wild type. 24-36h post transfection, cell lysates were prepared and subjected to immunoblotting with the indicated antibodies (A) and with anti flag M2 antibody (B) and (C)

It is possible that the hinge, besides determining NS1B binding, is also necessary for efficient conjugation to target proteins in human cells.

## 2.4 DISCUSSION

IFN α/β mediated anti-viral activities form a major line of host defense against invading viruses. Most viruses, including influenza viruses, have developed strategies to counteract the IFN response (Haller, Kochs et al. 2006), (Katze, He et al. 2002), (Levy and Garcia-Sastre 2001), (Garcia-Sastre 2001). The NS1 proteins of influenza viruses play a crucial role in these counter measures against IFN (Krug, Yuan et al. 2003). The NS1 protein of influenza A viruses (NS1A) mediates this protection through both its RNA binding domain and effector domain. The NS1A RNA binding domain inhibits the IFN induced 2'5' OAS- RNaseL pathway (Min and Krug 2006). The NS1A effector domain inhibits function of the cellular CPSF30, thereby inhibiting the processing of IFN pre-mRNA to form mature IFN mRNA in the cytoplasm (Nemeroff, Barabino et al. 1998), (Das, Ma et al. 2008), and also inhibits activation of PKR (Das, Ma et al. 2008), (Min, Li et al. 2007). In contrast, much less is known about how the NS1 protein of influenza B virus (NS1B) counteracts the actions of IFN. ISG15 is one of the most strongly induced genes by IFN α/β and is conjugated in an IFN dependent manner to hundreds of targets (Zhao, Denison et al. 2005). ISG15 and ISG15 conjugation have been shown to inhibit several viruses (Lenschow, Lai et al. 2007), (Lai, Struckhoff et al. 2009), (Okumura, Lu et al. 2006), (Okumura, Pitha et al. 2008), (Guerra, Caceres et al. 2008). Previously in our laboratory, NS1B was identified as a binding partner for ISG15 (Yuan and Krug 2001). In the current study, we show that a mutant influenza B virus which

encodes a NS1B that lacks ISG15 binding is attenuated in human but not canine cells, the reason being the species specific binding of ISG15 to NS1B. We further extend these results by identifying a species specificity determinant in ISG15 for binding NS1B. Our results demonstrate the anti-viral activity of ISG15 against influenza B, and also highlight the importance of human/primate cells to study influenza B virus and the interaction of its NS1B protein with ISG15.

### *2.4.1 ISG15 binding site on NS1B*

The N terminus of NS1B (amino acids 1-93) constitutes its dsRNA binding domain (Wang and Krug 1996). The structure of this domain is very similar to that of NS1A, and is in the form of a dimer, with each monomer contributing three alpha helices. Alpha helix two from both chains form a groove in which dsRNA binds (Yin, Khan et al. 2007). Previous mutagenesis data from our laboratory identified the loop between alpha helices one and two (loop1) to be the ISG15 binding site (Yuan, Aramini et al. 2002). However, subsequently it has been proven that the NS1B structure model on which those mutations were based were wrong and the mutations made in that study would disrupt the protein structure.

In our study, the identification of the ISG15 binding site on NS1B has uncovered a role for the helix 3 of NS1B as well as a role for amino acid 100. These results are consistent with previous data from our laboratory which had implicated both the region in between the RNA binding domain (amino acid 93) and amino acid 104 in the ISG15 binding site. We believe that the structure of the AAA mutant NS1B (which cannot bind ISG15) is largely intact since this mutant still retains other characteristics of the wild type

protein like its localization to nuclear speckles. Besides, a recombinant virus that encodes this mutant NS1B is not attenuated in MDCK cells, proving that the NS1B protein is still functionally intact. In contrast, a recombinant virus with the whole NS1B protein deleted shows extreme attenuation (Dauber, Heins et al. 2004).

### *2.4.2 Human cell culture systems for study of ISG15 and influenza B virus*

The predominant cell type used to study influenza viruses in tissue culture are MDCK cells, which are derived from a canine kidney source. However, our finding that NS1B does not bind canine ISG15 meant that we could not use this cell type to characterize the AAA mutant virus. Consistent with these results, the AAA mutant virus in not attenuated in MDCK cells. The influenza viral polymerase is extremely error prone and lacks proof reading function; therefore it is reasonable to hypothesize that ISG15 binding would not have been preserved if not serving a functional advantage to the virus. A sequence alignment of all the 466 NS1B sequences in the influenza resource in NCBI website (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) indeed found a high degree of sequence conservation of the ISG15 binding site (data not shown). These reasons prompted us to screen other human tissue culture systems that might be more suitable to characterize the AAA mutant virus.

The study of influenza viruses in tissue culture is limited by the fact that very few cell- types can support a viable multiple cycle growth of the virus. This limitation is due in part to the requirement of cleavage of the viral HA molecule for viable infection (Lazarowitz and Choppin 1975), (Klenk, Rott et al. 1975). HA cleavage can be carried out by either host furin-like or trypsin-like proteases (reviewed in (Webster and Rott

1987), (Steinhauer 1999)). Cleavage by the ubiquitous intra-cellular furin-like proteases requires a poly-basic cleavage site in HA, which is found in many highly pathogenic avian influenza A viruses. These viruses can achieve a multiple cycle growth in many cell types. Cleavage by extra-cellular trypsin-like proteases can occur with a mono basic cleavage site containing a single arginine in HA. Such a cleavage site is found in most human and non pathogenic avian influenza A viruses and all isolates of influenza B viruses. Propagation of viruses with mono basic cleavage sites in their HA in tissue culture require the exogenous addition of trypsin to the culture media, which very few cells can tolerate. Introduction of a poly-basic cleavage site into human influenza A viruses was able to render them susceptible to furin-like proteases (Kawaoka 1991), (Ohuchi, Ohuchi et al. 1991), although in some cases, the intracellular cleavage efficiency is low (Kawaoka 1991). We could not use a similar strategy for influenza B virus since a previous study had shown that introduction of a poly-basic cleavage site into the influenza B HA was unable to render the molecule cleavable by intra-cellular furin-like proteases (Brassard and Lamb 1997), suggesting that B/HA is intrinsically different from HAs of avian influenza A viruses.

In our screen to identify a suitable cell line to study influenza B viruses, only one cell type, Calu-3, was able to achieve viral titers comparable to MDCK cells, indicating a multiple cycle growth of the virus. These cells were previously shown to support growth of the 1918 influenza A virus (Tumpey, Basler et al. 2005). Interestingly, Calu-3 cells were able to support growth of influenza viruses both in the presence and absence of exogenously added trypsin (data not shown; (Tumpey, Basler et al. 2005)). It is possible that these cells encode (and secrete) a trpsin-like protease that is able to cleave HA.

In Calu-3 cells, the growth of AAA mutant virus was ten fold attenuated in early times post infection as compared to the wild type virus. This is in contrast to growth in MDCK cells where the AAA virus was not attenuated and in fact grew slightly faster (two fold) than wild type in early times post infection. One reason for the relatively modest attenuation could be that the AAA NS1B does not completely relieve the inhibition of ISG15 (and its conjugates) that is caused by the wild type protein (for detailed discussion on this, refer section 3.6.3). Hence, the attenuation of the AAA virus maybe due to the partial inhibition of ISG15 conjugation and also due to the inhibition of function(s) of free ISG15. Consistent with the latter possibility, ISG15 is translocated inside the nucleus during wild type B virus infection but not during AAA mutant virus infection. By mis-localizing ISG15, wild type NS1B might be inhibiting its interaction or conjugation to proteins. ISG15 is also secreted as a cytokine and has immune-modulatory functions (Recht, Borden et al. 1991), (D'Cunha, Knight et al. 1996), (Owhashi, Taoka et al. 2003). Our interpretation of preliminary experiments to study the secretion of ISG15 were complicated by the fact that we were not able to distinguish between secreted ISG15 and ISG15 that was released into the media due to cell death upon transfection. It will be interesting to test the effects of ISG15 secretion in a co-culture experiment with macrophages or NK T cells in presence and absence of NS1B. While suggesting an inhibition of free ISG15, our results also do not exclude a role for the inhibition of function(s) of pre-existing ISG15 conjugates by NS1B. It is possible that NS1B also sequesters pre-existing ISG15 conjugates into nuclear speckles. Indeed, our studies show that NS1B is able to bind not only free ISG15 but also the conjugated form. When

influenza B virus infects cells where ISG15 conjugates are already present, their nuclear sequestration might result in inhibition of the function(s) of ISG15-conjugated protein(s).

### *2.4.3 Effect of NS1B on ISG15 conjugation*

Several lines of evidence suggest that NS1B reduces the levels of ISG15 conjugation. We have observed that there is little to no ISG15 conjugation present in wild type influenza B infected cells. Also, transfection of a NS1B expressing plasmid reduces the intra-cellular levels of ISG15 conjugates in IFN treated cells and also in cells co-transfected with plasmids expressing the three ISG15 conjugation enzymes, UBE1L, UBCH8 and HERC5. Previous studies in our lab using UBE1L expressing insect Sf9 cell extracts showed that NS1B is able to inhibit the formation of the UBE1L~ISG15 thioester bond (Yuan and Krug 2001). However, we and other groups (Chang, Yan et al. 2008) have subsequently been unable to repeat this result using an *in vitro* assay using purified UBE1L. We also have found that NS1B is similarly unable to inhibit the second step in the pathway, i.e UBCH8~ISG15 thiol ester bond formation. The C terminal domain of ISG15 is sufficient to form thiol ester bonds with both UBE1L and UBCH8, while the N terminal domain is involved in increasing the repertoire of target conjugates presumably by recognizing the ISG15 ligase (Chang, Yan et al. 2008). Since NS1B binds ISG15 on its N terminus, we hypothesize that NS1B interferes with the last step in the conjugation pathway, which involves HERC5. The exact mechanism of the inhibition remains unresolved. The inhibition could either be at the step of HERC5~ISG15 thiol ester formation or at the level of target recognition by HERC5. Currently an *in vitro* system for the detection of HERC5~ISG15 thiol ester bond formation is unavailable

because of the difficulty in purifying biologically active HERC5 protein (Dastur, Beaudenon et al. 2006). Development of such an assay would help in testing this hypothesis directly.

Surprisingly, we found that binding of ISG15 is not the sole mechanism by which NS1B inhibits ISG15 conjugation. Two experiments support this conclusion. First, when a plasmid expressing the AAA mutant NS1B is co-transfected with plasmids expressing the enzymes of the ISG15 conjugation system, only a partial recovery of ISG15 conjugation is observed. Second, wild type NS1B is able to partially suppress the conjugation of an ISG15 (hinge) mutant that lacks binding capability to NS1B. These results indicate that the full effects of NS1B on the ISG15 conjugation system are not completely reversed by simply knocking down ISG15 binding function of NS1B. These observations explain why the replication of the AAA mutant virus in Calu-3 cells was only ten fold slower than that of the wild type virus. Future experiments will be directed at identifying the NS1B sequence(s) that mediate the complete inhibition of ISG15 conjugation, and determining whether a influenza B virus expressing such a mutant NS1B protein is more attenuated than the AAA mutant described here.

### *2.4.4 Species specific interaction of ISG15 with NS1B*

A significant finding in our research is that the binding between ISG15 and NS1B is species specific. NS1B binds human and African green monkey ISG15 proteins but not mouse or canine ISG15 proteins. We also identified the hinge region between the N and C terminal ubiquitin-like domains of ISG15 as the principal species specific determinant

for NS1B binding. In addition to forming possible contacts with NS1B, the hinge region in human and monkey ISG15 may be orienting the two ubiquitin-like domains in such a way as to allow NS1B access to the binding site on the N terminal domain, while in canine and mouse ISG15 proteins, the orientation of the two ubiquitin-like domains might block NS1B from the binding site. In fact, our results suggest that at least part of the binding pocket is conserved between species and is composed of the β sheet immediately preceding the hinge region on the N terminal domain. Another surprising finding in our study is that the human ISG15 with hinge from mouse ISG15 is not only able to inhibit NS1B binding, but also reduces the mutant's ability to conjugate to target proteins. Other studies have proposed that the N terminal region of ISG15 is responsible for recognizing HERC5 (Chang, Yan et al. 2008), thereby facilitating ISG15 conjugation. However our results suggest that the hinge plays a large role in this process.

ISG15, unlike ubiquitin, is not very well conserved among different species and shows wide variation in sequence. Many genes involved in innate immunity show great divergence between species due to evolutionary pressure. For example, TRIM5α is a protein that inhibits HIV-1 infection by interfering with virus un-coating in old world monkeys but not in humans (Stremlau, Owens et al. 2004). The HIV protein Vif is able to counter act another anti-viral protein APOBEC3G in humans and chimpanzees but is ineffective against mouse, rhesus macaque and African green monkey orthologs. Similarly, SIV Vif is able to inhibit African green monkey but not human APOBEC3G (Mariani, Chen et al. 2003). Paramyxoviruses also show a differential ability to inhibit the IFN response in a species specific manner. STAT-2 is degraded by paramyxovirus simian virus 5 (SV5) in human but not the mouse cells, leading to the inability of virus to

inhibit IFN α/β signaling in mice resulting in virus restriction in this organism (Parisien, Lau et al. 2002). Influenza B virus has been isolated from only humans, and canines and mice are not natural hosts. Consequently, it is not surprising that the NS1B protein has evolved to bind specifically to human ISG15 (and its primate progenitors), but not to canine and mouse ISG15 proteins. For this reason, mouse and canine ISG15 proteins would be expected to be effective in their anti-viral activities against influenza B virus since NS1B does not bind these molecules. Indeed this is what was found when influenza B virus was rescued in ISG15$^{-/-}$ mice (Lenschow, Lai et al. 2007), suggesting that in these animals, NS1B was ineffective in countering the anti-viral properties of ISG15. Recently, it has also been shown that influenza B virus is rescued in UBE1L $^{-/-}$ mice (Lai, Struckhoff et al. 2009). At present, it is not known if NS1B inhibits conjugation of mouse ISG15 in mouse cells. Taken together, our studies highlight the unsuitability of mouse models of ISG15 as well as the commonly used MDCK cells to study influenza B virus.

Since NS1B binds the more closely related monkey ISG15, we propose old world monkeys (owm) such as rhesus macaques as a more suitable animal model to study influenza B virus. Owm ISG15 was recently shown to conjugate much stronger than either human or mouse. This is due to the ability of owm ISG15 to be conjugated by the E1 enzyme of ubiquitin (Pattyn, Verhee et al. 2008). A suitable 'owm' cell line which is able to support multiple cycle growth of influenza B virus could be a valuable tool in better understanding the interplay between ISG15 and influenza B virus. One African green monkey cell line, Vero, has been used to grow high titers of influenza viruses. However, these cells do not respond to IFN since they lack IFN genes. Treatment of these cells with Poly I:C also did not induce much ISG15 (data not shown). Another African

green monkey cell line Cos7 induced ISG15 upon treatment with Poly I:C but was not able to reach high titers of virus growth. Further screening will be important to identify a monkey cell line that has a robust IFN response as well as the ability to support multiple cycle influenza B virus growth.

Another line of useful experiments would be to adapt the MDCK cells to study influenza B virus, for example, by expressing human ISG15 in these cells to see if the AAA mutant virus is attenuated. However, high transfection efficiencies could not be achieved in MDCK cells and our efforts to make a stable MDCK cell line to constitutively express human ISG15 proved toxic to the cells. Hence, it might be necessary to make a stable cell line with ISG15 expression under an inducible promoter. An alternative could be to engineer the virus itself to over express ISG15 in the infected cells. A similar strategy was used for Sindbis virus to show the sensitivity of that virus to virally expressed ISG15 (Lenschow, Giannakopoulos et al. 2005). While influenza A and B viruses contain eight genomic segments, influenza C virus contains only seven, since in the latter's case, the functions of both the HA and NA are carried out by a single enzyme HEF. Recently, a recombinant influenza A virus was generated that contained the C/HEF protein, thereby allowing the expression of a reporter GFP plasmid from the NA segment (Gao, Brydon et al. 2008). Theoretically, such an approach should also work for influenza B virus, and would afford a system in which ISG15 or any other protein of interest could be expressed from the virus itself.

Structural studies are required to provide insights into the exact mode of binding between ISG15 and NS1B. It would be interesting to see if a small region of human ISG15 would be able to assume a three dimensional structure to bind NS1B. If so, then

we could test to see if over expression of that fragment of ISG15 will be enough to inhibit influenza B virus. Such an experiment has precedence in influenza A virus where a small fragment of the cellular CPSF 30 protein was expressed in cells to inhibit the virus (Twu, Noah et al. 2006). Such strategies could have future therapeutic implications to inhibit influenza B viruses.

In summary, my research has identified important determinants of the interaction between NS1B and ISG15. My results provide the first example of a species (human) specific function for a protein encoded by this predominantly human influenza B virus. It also raises exciting possibilities about differences in functions of the ISG15 system in different species. Future experiments will nevertheless be important to further characterize the different levels of regulation of NS1B on the ISG15 system and the effect of this regulation on influenza B virus replication.

# CHAPTER 3: Studies on the intra-nuclear localization of the influenza B virus NS1B protein

## 3.1 INTRODUCTION

Influenza viruses are unique among other negative strand segmented RNA viruses in that they replicate in the nucleus of the infected cell. Therefore, the viral RNA along with proteins involved in its replication (like viral polymerase complex and NP) must be transported inside the nucleus. After replication is complete, the viral RNP complex must be exported out of the nucleus with the help of the NS2 protein. Therefore, many of the influenza viral proteins contain nuclear localization signals that traffic them to the nucleus (reviewed in (Boulo, Akarsu et al. 2007)).

NS1 proteins of influenza viruses are multi-functional proteins. NS1 of influenza A virus (NS1A) has been characterized in depth and has been found to play a wide range of roles during infection, from inhibition of host anti-viral responses to regulating viral polymerase function (reviewed in (Hale, Randall et al. 2008)). Some of these functions are presumably carried out in the cytoplasm (like inhibition of PKR); however other functions are carried out in the nucleus. Indeed, a crucial function of NS1A is to inhibit the processing of host pre-mRNAs, thereby preventing the nuclear export of mature host mRNAs (Noah, Twu et al. 2003), (Das, Ma et al. 2008). NS1A carries out these functions in the nucleus by binding two nuclear proteins, CPSF30 and PABII. These factors are involved in 3' end processing of cellular mRNAs, and their functions are inhibited by binding to NS1A (Nemeroff, Barabino et al. 1998), (Chen, Li et al. 1999). Nuclear

targeting of NS1A from human viruses is mediated by two nuclear localization signals (NLSs) present in the protein (Greenspan, Palese et al. 1988). NLS1 is a classic mono-partite signal (consisting of 4-6 basic amino acids), and is present within the N terminal dsRNA binding domain which also coincides with the binding site for dsRNA (R38 and K41). NLS2 is present in the C terminal effector domain (amino acids 219-230). Inside the nucleus, NS1A is mostly present in the nucleoplasm, but also accumulates inside the nucleolus (NLS2 also functions as a nucleolar localization signal (NoLS)). Both NLS1 and NLS2 mediate binding to importin α, and it has been shown that NS1A binds all six isoforms of importin α (Melen, Kinnunen et al. 2007).

NS1B protein of influenza B virus also contains a monopartite NLS which overlaps with its dsRNA binding region, and we and others have shown that the NS1B protein is imported into the nucleus (Schneider, Dauber et al. 2009). However unlike NS1A, it lacks a NLS/NoLS in the C terminus. While at least some of the nuclear functions of NS1A have been elucidated (namely inhibition of 3' end processing of mRNAs, regulation of viral polymerase function), little is known about the nuclear function(s) of NS1B. In fact, the only known interacting partner for NS1B is the cellular factor ISG15, which is a cytoplasmic protein (Loeb and Haas 1994). Therefore, much research is required to understand the nuclear functions of NS1B. To begin to understand NS1B's nuclear functions, we carried out studies on the localization of NS1B. We found that NS1B is localized to the nucleus during early stages of infection while at later stages is present in the cytoplasm. Inside the nucleus, it accumulates in intra-nuclear compartments called SC35 speckles. This is a unique property of NS1B as it is not shared with NS1A. Splicing or SC35 speckles are so called because of their characteristic

accumulation of the splicing factor SC35. They are also enriched in other splicing factors, along with RNA and other proteins involved in RNA metabolism (reviewed in (Lamond and Spector 2003)). We show that targeting of NS1B to speckles does not require other viral proteins and is not RNA mediated. Furthermore, we found that nuclear speckle localization of NS1B is mediated by sequences in both the N-terminal RNA-binding domain and the C terminal effector domain.

## 3.2 MATERIALS AND METHODS

### *Cell lines*

MDCK, A549 and Hela cells were cultured in DMEM (GIBCO®) supplemented with 10% fetal bovine serum (GIBCO®) and 1% PSG (penicillin streptomycin glutamate) at 37ºC with 5% $CO_2$ /95% air atmosphere.

### *Plasmids and transfection*

GFP tagged NS1B plasmids were made by cloning the DNA encoding NS1B into the pEGFP-C1 plasmid (Clonetech) using Bgl2 and Hind3 restriction sites on the vector. For insertion of NLS from the large T antigen of SV40 virus (SV40-NLS), the NLS was added to the N terminus of NS1B by a two step PCR reaction where the 5' primer in the first step of PCR contained part of the sequences of the NLS overlapped with the 5' primer used during the second step of the PCR. The DNA for NLS-NS1B was then cloned downstream of GFP in the pEGFPC1 vector using Bgl2 and Hind3 restriction

enzyme sites. The plasmids encoding GFP-NS1B were transfected into Hela cells in using Mirus transfection reagent following the manufacturer's protocol.

***Viruses and infection***

Wild type influenza B/Yamanashi/1998 virus was generated by reverse genetics and amplified in ten day old embroynated chicken eggs as described in chapter 2 (refer materials and methods). N terminal 3xflag tagged NS wild type influenza B virus was generated by J.W. Park (see chapter 2, materials and methods for details) and amplified in MDCK cells.

***In vitro dsRNA binding assay***

***GST NS1B protein(s) purication:*** For purification of GST NS1B proteins in bacteria, DNA for NS1B 1-104, R50A, R53A and R50AR53A in 1-104 background were cloned into pGEX4T2 vector using BamHI and XhoI sites on the vector and Bgl2 on 5' end and XhoI on 3' end of the insert. The proteins were then expressed and purified from a 1 litre culture of *E.coli* as described (chapter 2, materials and methods).

***Probe preparation****:* A 55bp β globin template was generated by PCR from a 210bp β globin template, such that the PCR product had sites for the restriction enzymes BamH1 or EcoR1, as well as T7 or SP6 polymerase site on each end. An EcoR1 or BamH1 digest of the PCR product was used as template for transcription by SP6 or T7 polymerase (Invitrogen) respectively. $^{32}$P labeled single stranded RNA were generated by incorporation of $^{32}$P labeled UTP in the *in vitro* transcription by the RNA polymerase in a 30µl reaction. To form dsRNA, the transcripts (in 10mm HEPES, pH7.6 and 50mM

NaCl) were heated to 90ºC for two minutes and allowed to anneal to each other by slow cooling to room temperature.

***dsRNA binding assay:*** Increasing concentrations (0.5, 1 and 1.5 µg) of NS1B wt, R50A, R53A or R50AR53A in 1-104 background that were purified from *E.coli* and GST tag cleaved off were incubated with 5 µl of $^{32}$P labeled dsRNA in the presence of binding buffer (50mM Tris-Cl pH 8.0, 50mM KCl, 50mM glycine, 2.5mM DTT, o.5 units/µl RNasin (Promega) and 50mg/ml of *E.coli* tRNA (Promega)) for 30 minutes at room temperature. The reaction was stopped by addition of 6X RNA loading buffer and the components separated on a 6% non-denaturing polyacrylamide gel. The labeled probe and its complexes were detected by autoradiography.

***Immunofluroscence***

Immunofluroscence was performed as described in materials and methods (chapter 2).

## 3.3 RESULTS

### 3.3.1 NS1B shows unique sub-cellular localization

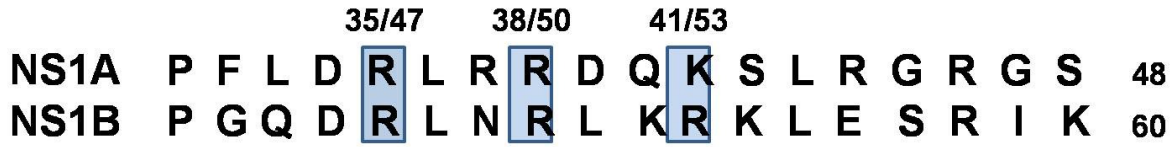
NS1A is a nuclear protein and has been shown to accumulate inside the nucleolus at certain times during infection. NS1B contains a nuclear localization signal (NLS) in its N terminal RNA binding domain that is analogous to NLS1 in NS1A (figure 3.1A, boxed residues). We therefore tested to see if NS1B also localizes to the nucleus. NS1B localization was observed during infection of MDCK cells using anti NS1B antibody. NS1B does enter the nucleus and interestingly, it showed distinct dot-like accumulation (figure 3.1B). We have shown that NS1B has some species specific functions, namely

binding to human but not canine ISG15 (refer chapter 2). Since influenza B virus is predominantly a human virus, we wanted to confirm that this intra nuclear localization was not unique to MDCK cells but was also observed in human cell lines. The polyclonal NS1B antibody has high background during immunofluroscence; therefore we utilized a recombinant B/Yamanashi/1998 virus which expresses a NS1B with a 3xflag tag at its N terminus (developed by J.W. Park). Such N terminal tagging is not detrimental to the virus, and the NS1B protein is efficiently detected when immunoblotted with anti flag antibody (data not shown). Immunofluroscence with anti flag antibody showed that the 3xflag tagged NS1B showed similar intra nuclear localization in both A549 and MDCK cells (figure 3.2). Similar localization was also observed in Hela cells (chapter 2, figure 2.4). Hence, unlike ISG15 binding which is species specific, the intranuclear localization of NS1B is conserved across species. Further, it was observed that at early times post infection (5h), NS1B is nuclear (figure 3.2A), but at later times (9h), becomes predominantly cytoplasmic (figure 3.2B). This is very unlike NS1A protein which is nuclear for most of the influenza A viral life cycle.

### 3.3.2 NS1B localizes to SC35 speckles due to an intrinsic property of the protein

Next, we wanted to determine if the intra-nuclear localization of NS1B is intrinsic to the protein or requires the action of other viral proteins. To test this, a plasmid expressing NS1B with a N terminal GFP tag was constructed and transfected into Hela cells. While the control GFP protein was distributed all over the cell (figure 3.3A), GFP-NS1B was nuclear and accumulated in speckled regions within the nucleus (figure 3.3B).

A




B

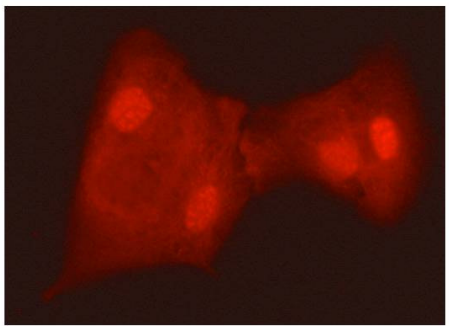


**Figure 3.1 NS1B is a nuclear protein** A.A sequence alignment of amino acids 31-48 of NS1A and 43-60 of NS1B. Boxes represent amino acids in NS1A (R35, R38 and K41) that are required for importin-α binding and the corresponding residues in NS1B. B. MDCK cells were infected with wild type influenza B/Yamanashi/98 virus. 5h post infection, cells were fixed and subjected to immuno-fluroscence with anti NS1B antibody. Secondary antibody used was anti rabbit conjugated to TRITC.

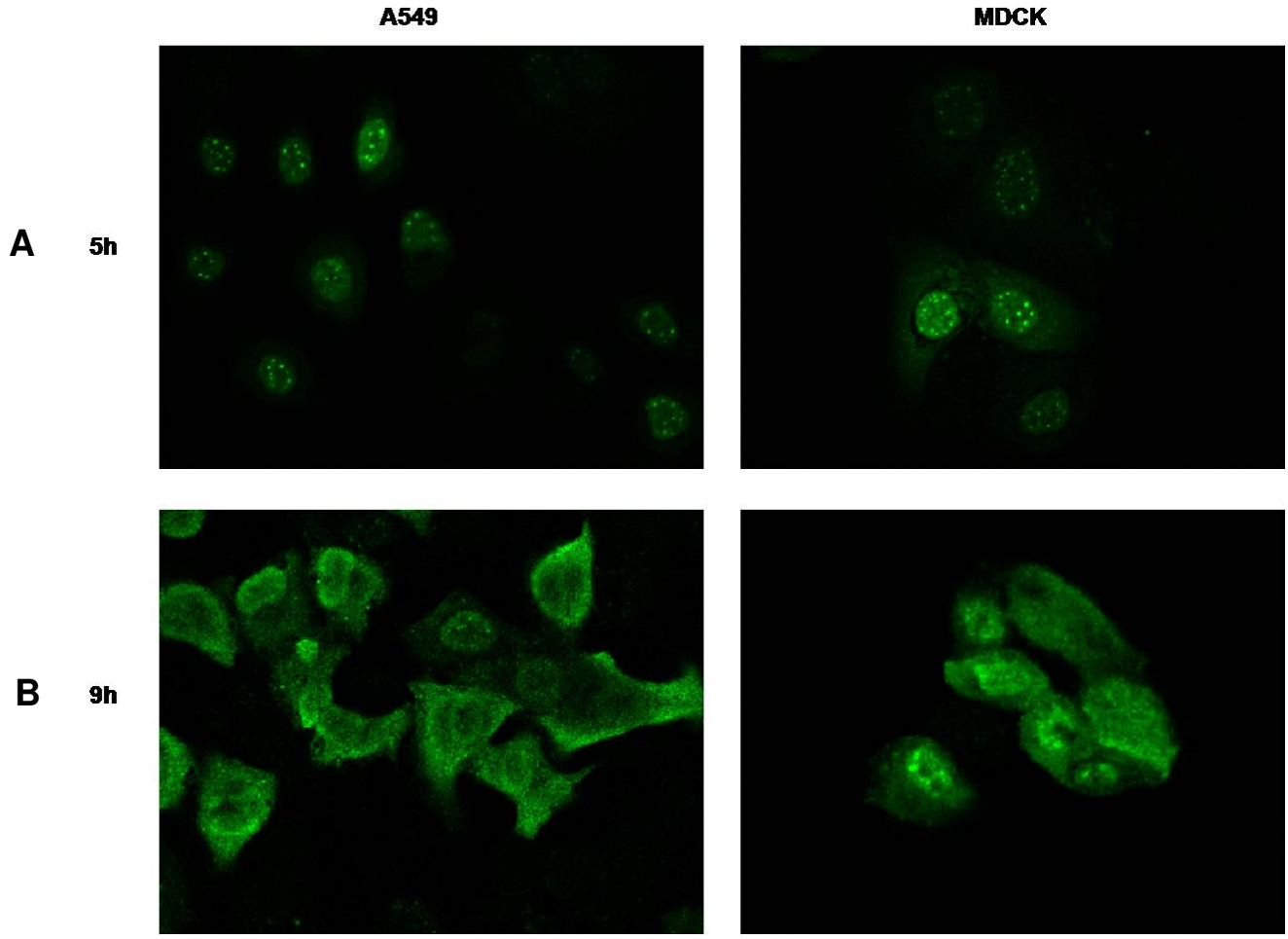



**Figure 3.2 NS1B localizes to nucleus during early phases of infection.** A549 or MDCK cells were infected with N terminal 3xflag tagged wild type NS influenza B virus. 5 (A) or 9 (B) hours post infection, cells were fixed and subjected to immunofluroscence with mouse anti flag antibody to detect NS1B. Secondary antibody used was anti mouse antibody conjugated to FITC.

The nucleus contains many sub-nuclear compartments which are identified by the presence of characteristic proteins. Immuno-fluroscence with an antibody that detected the splicing factor, SC35, showed a high degree of overlap with the GFP-NS1B signal (figure 3.3B, merge panel). These results prove that NS1B localizes to SC35 or splicing speckles and that this localization does not depend on the actions of other viral proteins. SC35 speckles are alternatively called splicing speckles and are enriched in splicing factors as well as RNA binding proteins, RNA polymerase II subunits as well as other RNA processing factors. Many of these factors are found in more than one nuclear sub-compartment; however SC35 characteristically accumulates in splicing speckles and are excluded from other compartments like para-speckles.

### 3.3.3 RNA binding property of NS1B is not required for speckle localization

NS1B is an RNA binding protein and shares a structurally conserved N terminal dsRNA binding domain with NS1A. Splicing speckles are comprised of over 130 proteins, many of which are RNA-binding proteins and are involved in various steps of RNA metabolism. Besides, speckles also contain a stable population of RNA. These reasons led us to test if the dsRNA binding property of NS1B is responsible for its speckle localization.

#### *3.3.3.1 Residues required for dsRNA binding of NS1B*

Earlier studies on the dsRNA binding domain of NS1B had identified the residues R50 and R53 as important for dsRNA binding in an *in vitro* assay. Sequence alignment

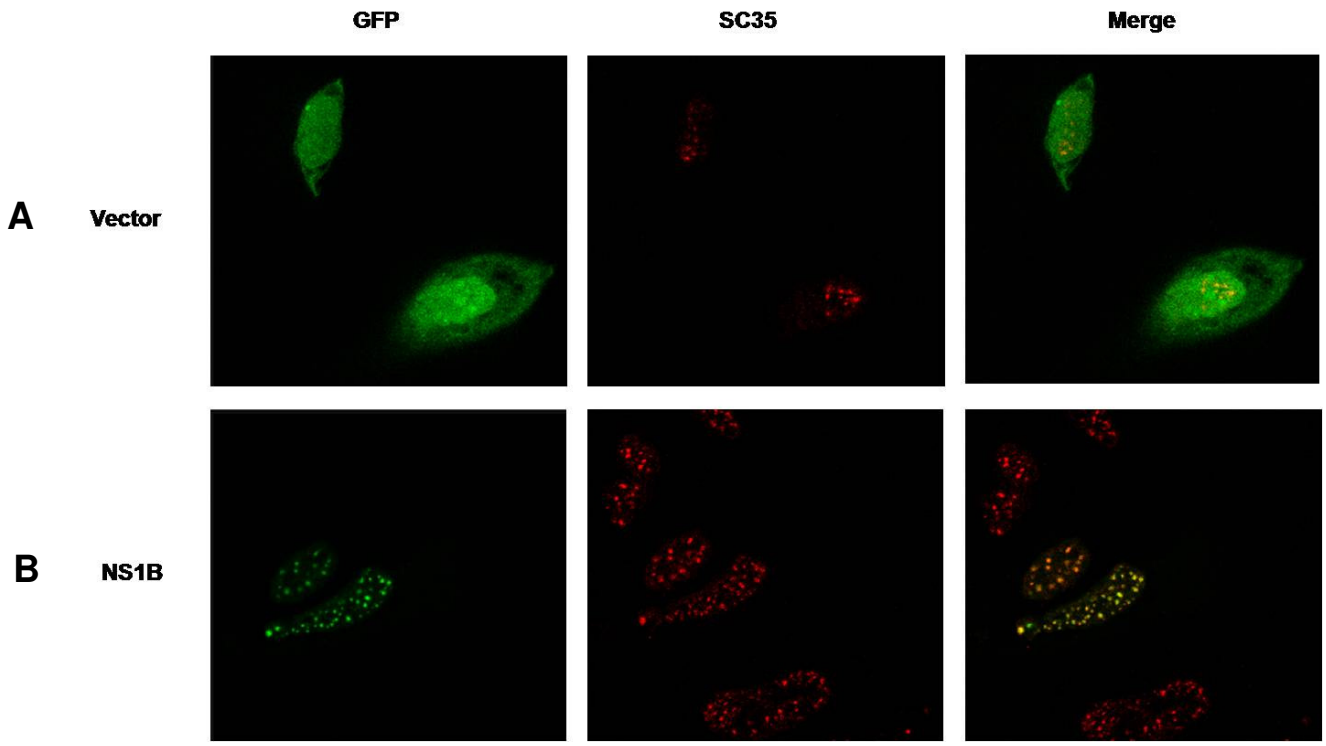



**Figure 3.3 NS1B localizes to SC35 speckles.** Hela cells were transfected with GFP empty vector (A) or GFP NS1B (B). 24h post transfection, cells were fixed and immunofluroscence was conducted with anti SC35 as primary antibody and anti mouse conjugated to TRITC as secondary antibody. GFP signal was detected with UV light. The merge panel shows the overlap of GFP and TRITC signals. Yellow color denotes colocalization.

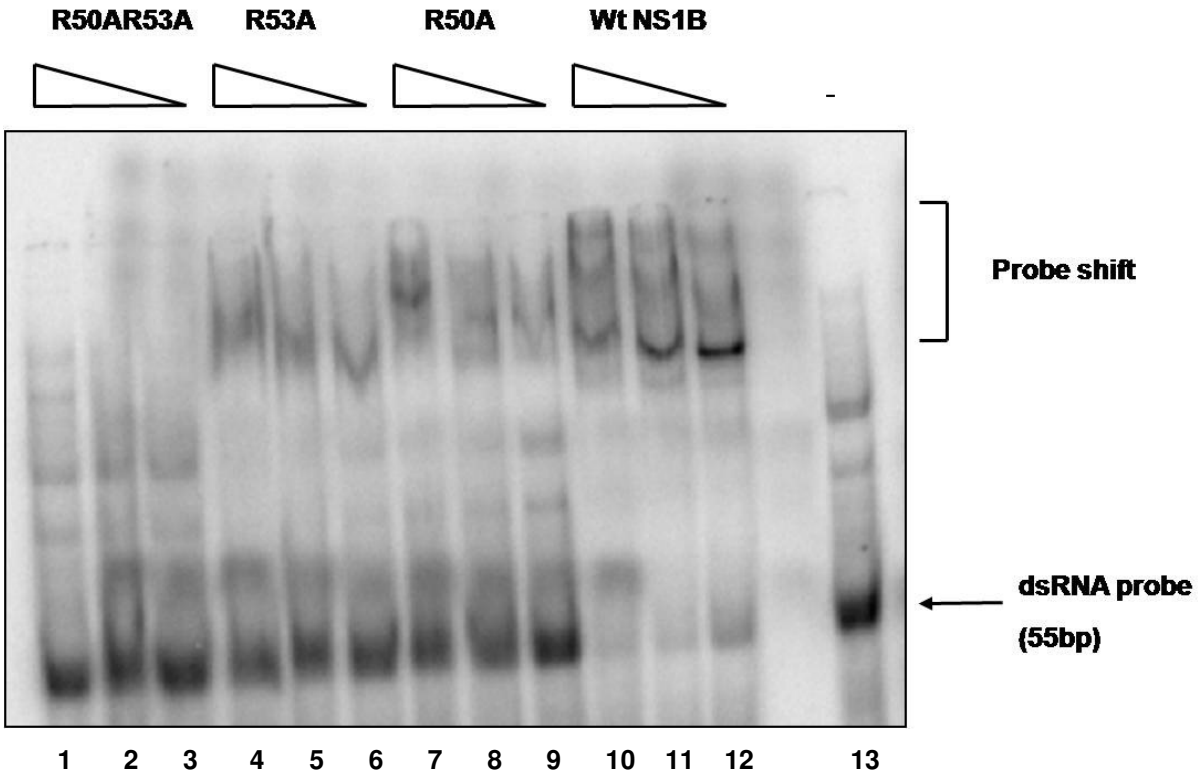



**Figure 3.4 Residues required for dsRNA binding of NS1B.** 5µl of $^{32}$P labeled dsRNA probe (lane 13) was incubated with decreasing concentrations of bacterially purified GST NS1B wild type (lanes 10-12), R50A (lanes 7-9), R53A (lanes 4-6) or R50AR53A (lanes 1-3) proteins for 30 minutes at RT. Reactions were stopped by adding 6X loading buffer and complexes were separated on a 6% non denaturing acrylamide gel. The radiolabeled probe was detected by autoradiography.

and structural studies of NS1A and NS1B predict that R50 and R53 of NS1B are analogous to R38 and K41 of NS1A respectively. A crystal structure of the RNA binding domain of NS1A bound to RNA shows that R38 penetrates the RNA helix and is the most important residue for dsRNA binding. Consistent with this prediction, a single mutation R38A in NS1A is sufficient to completely disrupt dsRNA binding of the wild type protein. Based on these data, we wanted to test if a single mutation of either R50 or R53 of NS1B is sufficient to disrupt its dsRNA binding. Bacterially expressed GST NS1B wild type, R50A, R53A or double mutant R50AR53A were incubated with a $^{32}P$ labeled 55bp dsRNA generated by *in vitro* transcription from a β globin template (see Materials and Methods). The resulting complexes were separated in a native poly-acrylamide gel, and the migration of the radio-labeled dsRNA was detected by autoradiography. NS1B wild type protein shifted all the dsRNA into a slower migrating complex (figure 3.4, lanes 10-12). Although most of the dsRNA was unbound when incubated with either of the single mutant, R50A or R53A, weak binding could still be detected (figure 3.4, lanes 4-9). In contrast, the double mutant R50AR53A NS1B lacked detectable dsRNA-binding activity as most of the probe remained unbound (figure 3.4, lanes 1-3). These results indicate that for full knockdown of dsRNA binding capacity of NS1B, both these residues must be mutated.

#### *3.3.3.2 Localization of dsRNA binding mutant of NS1B*

A plasmid expressing GFP-NS1B R50A/R53A was transfected into Hela cells (figure 3.5A). Since this region also encodes the nuclear localization signal, most of the

protein was mis-localized to the cytoplasm. Some of the protein still entered the nucleus and accumulated in speckles. To conclusively see if this mutant localized to speckles, a NLS sequence was fused at the N terminus. The NLS from SV40 large T antigen is a classical monopartite nuclear localization signal and has been shown to function in 'trans' when attached to any reporter protein. We inserted the NLS between the GFP and the NS1B R50A/R53A sequence as shown in figure 3.5B. The GFP-NLS-R50AR53A protein was efficiently transported into the nucleus and accumulated inside speckles (figure 3.5B), proving conclusively that dsRNA binding is not responsible for mediating speckle localization of the NS1B protein.

### 3.3.4. Both N terminal and C terminal domains of NS1B contribute to speckle localization

In some proteins, the nuclear and speckle localization signals cannot be separated. However, our finding that even upon mutation of the nuclear localization signal, NS1B accumulates in the speckles when supplied with an NLS proves that in NS1B at least, the two signals can be separated. To identify the region of NS1B that is responsible for speckle localization, we first used plasmids expressing either GFP-NS1B (1-93) or GFP-NS1B (1-104), and observed the localization of the tagged proteins inside the cell (figure 3.6A). Since these fragments contain the NLS, they are predominantly nuclear in localization. The nuclear proteins accumulated in speckles, but some of these proteins were also in the nucleoplasm. Localization of these two NS1B fragments to the speckles was less complete than observed for the full-length NS1B protein (compare with figure 3.3).

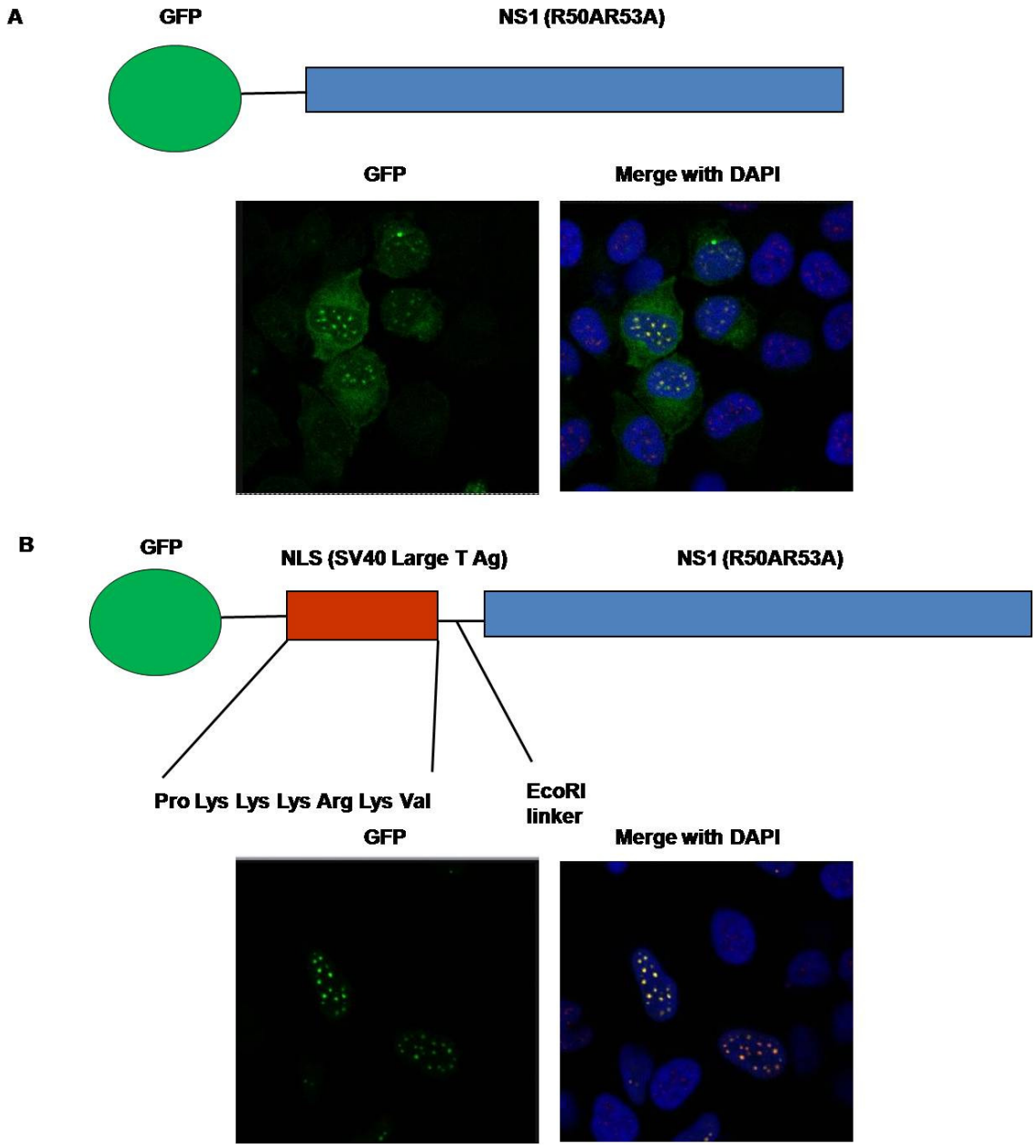



**Figure 3.5 Localization of dsRNA binding mutant of NS1B.** Hela cells were transfected with GFP tagged NS1B (R50AR53A) (A) or GFP-NLS-NS1B(R50AR53A) (B). 24h post infection cells were fixed and stained with DAPI to observe the nuclei or observed under UV light for GFP fluroscence. Merge panel shows overlap of the two signals.

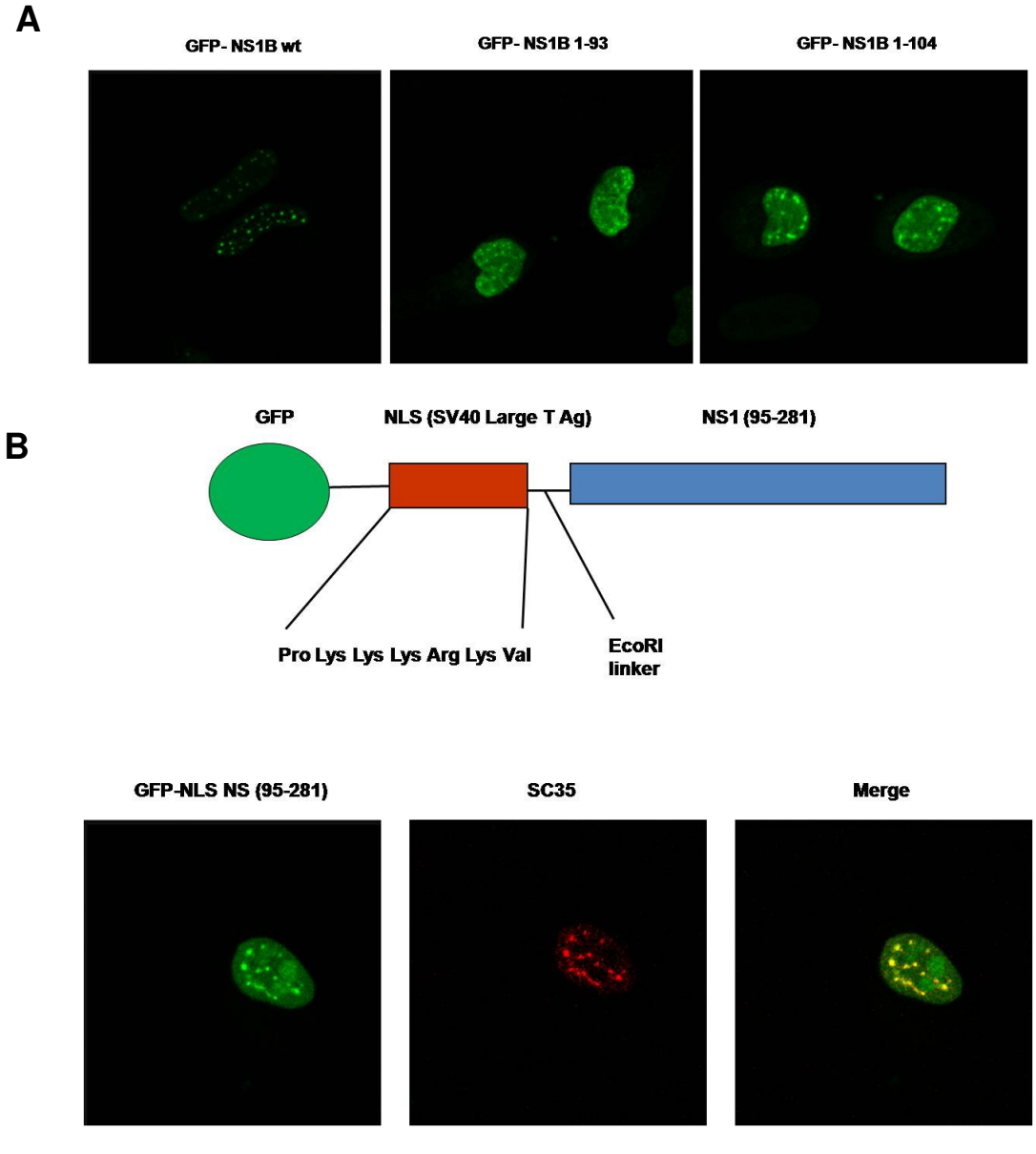



**Figure 3.6 Both N and C terminal domains of NS1B contribute to speckle localization.** Hela cells were transfected with the indicated plasmids expressing GFP tagged NS1B constructs. 24h post transfection, cells were fixed observed for GFP fluroscence under UV light. To observe SC35 speckles, fixed cells were subjected to immunofluroscence with mouse anti SC35 primary antibody and anti mouse secondary antibody conjugated to TRITC. Merge panel shows overlap of signals from GFP and TRITC fluroscence.

These results suggested that the C-terminal effector domain of NS1B also contributes to the localization to nuclear speckles. The NS1B effector domain lacks a NLS, and as result. GFP-NS1B (95-281) accumulates in the cytoplasm (data not shown). Therefore, we added the NLS from SV40 large T antigen between GFP and NS1B (95-281) (figure 3.6B). This protein localized to the nucleus, and showed slight enrichment in speckles (figure 3.6B). However, the localization of this NS1B domain was poor compared to the full length NS1B protein. Our results taken together suggest that speckle targeting signal is contributed by regions within both N and C terminal domains of NS1B protein.

## 3.4 DISCUSSION

The nucleus is a highly compartmentalized structure as shown by electron microscopy, with certain regions more electron dense as compared to the surrounding nucleoplasm. Several of these intranuclear compartments have been described in the literature, e.g., nucleolus, cajal bodies (CBs), promyelocytic leukemia (PML) bodies, gems, paraspeckles and splicing speckles (reviewed in (Spector 1993)). Each of these compartments has a complex composition and is composed of many different proteins and RNA populations. SC35 or splicing speckles are enriched in splicing factors like the SR protein SC35, and along with other RNA processing factors (Saitoh, Spahr et al. 2004). The function(s) of SC35 speckles have been the subject of debate, but it is now widely accepted that they act as storage/assembly sites for splicing/RNA processing factors from where the latter are recruited to sites of active transcription or splicing

(reviewed in (Lamond and Spector 2003)). Speckles contain more than 130 proteins as identified by mass spectrometry (Saitoh, Spahr et al. 2004). Analysis of these proteins did not reveal any single sequence or domain that was responsible for speckle localization. Several different targeting signals have been reported; in certain cases, specific sequences like the SR domain of certain SR proteins have been shown to translocate non-speckle localizing proteins into speckles when attached to them (Hedley, Amrein et al. 1995).

It is intriguing that the NS1B protein is found in speckles. Unlike NS1A, NS1B has no documented effect on RNA processing or splicing. Indeed, NS1A binds PABII, which is a speckle associated protein; however it is not found in speckles (Chen, Li et al. 1999). It is probable that NS1B is binding some protein that is localized to speckles. One of the ways to understand the function of speckle localization of NS1B would be to study the characteristics of a mutant protein that can no longer associate with speckles. Study of a recombinant virus that encodes such a mutant would be able to give insights as to whether speckle localization of NS1B is important to the virus. However, our efforts to identify a specific region that is responsible for speckle targeting have so far been unsuccessful. Our experiments suggest that both the N and C terminal domains of NS1B are involved in targeting since each alone only retains weak association with speckles. It is possible that in the three dimensional structure, a single surface that is responsible for localization has contributions from both the domains. Alternatively, there could be two separate surfaces that are additive and together mediate efficient localization. Our data suggest that the site(s) on both the domains need to be mutated together to completely eliminate speckle localization. However, our efforts to identify the region within the N terminal domain were not successful. Also, currently the structure of the C terminal

domain or full length NS1B is not available; therefore any mutation that is made in that domain carries the risk of disrupting the protein structure.

In the event that sites of NS1B that mediate speckle localization are not identified, other approaches might be taken to understand the reason for this unique localization property. One such approach could be to identify the protein factor(s) to which the NS1B protein is binding to in the speckle. A biochemical strategy for the purification of speckles from mouse liver has been developed and has been used to identify the protein composition of speckles through mass spectrometry (Mintz, Patterson et al. 1999). It might be useful to do an *in vitro* affinity pull down of an extract of the mouse liver speckles using the RNA binding mutant of NS1B to see if any protein(s) are pulled down by NS1B. Since the speckle localization of NS1B is conserved across species, this mouse extract is probably suitable for identifying the speckle interacting protein(s). Once such interacting speckle proteins are identified, it could be tested as to whether NS1B influences their function in any way.

It will also be interesting to see if other influenza B viral proteins like the polymerase or the viral RNA itself localizes to the speckles. It has been observed that the small antigen of the hepatitis delta virus is recruited to the speckles to aid viral RNA transcription (Bichko and Taylor 1996). It might be that NS1B has as yet unidentified functions in the splicing of viral mRNAs that leads to its association with host splicing factors. In addition, it is possible that NS1B localizes to the speckles to inhibit the action of certain host proteins that carry out antiviral functions. This is analogous to the situation with the NS1A protein, which inhibits 3' end processing of host pre-mRNAs so that antiviral proteins are not made (reviewed in (Krug, Yuan et al. 2003)). Another

possibility is that NS1B, by binding to certain speckle or RNA processing factors is hijacking or regulating a host pathway for the benefit of the virus. Such hijacking of cellular processes is a well known strategy used by viruses to aid their replication.

This study has clarified important questions that were not answered by a previous study. For example, my research has shown that the speckle localization signal can be distinguished from the NLS, does not depend on dsRNA binding of NS1B and is contributed by both N and C terminal domains of NS1B. Together, the results presented in this study have further enhanced our knowledge of the functions of NS1B protein. Nevertheless, future research is needed to uncover the role(s) of NS1B in the nucleus, thereby providing further insight into the replication strategies of influenza B virus.

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